REMARKS

Receipt of the Office Action mailed July 29, 2010 is hereby acknowledged. Applicant thanks the Examiner for the withdrawal of the rejections under 35 U.S.C. §§ 102(b) and 112. Reconsideration and withdrawal of the remaining rejections is respectfully requested.

Applicant has corrected Table 6 on page 34 of the specification to correct a typographical error and properly reflect that the last column of the table presents data from SuppM2, not SuppM1. This amendment is supported by the discussion of the data in the specification at pages 33-34.

Applicant has added new claim 71, which is supported in the specification at page 15, lines 29-32.

No new matter has been added.

Obviousness Rejection over Wachtel

The Examiner has rejected claims 57, 59, and 61-70 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Wachtel, et al., DE 4037447 ("Wachtel"). The Examiner admits that Wachtel teaches a different composition from the presently

claimed invention because Wachtel's composition have a higher amount of lysine. However, the Examiner asserts that it would merely have required routine optimization to arrive at the presently claimed invention from Wachtel. Applicant traverses.

As discussed in previous amendments, the claimed invention is an LNAA supplement with the following characteristics:

(1) the following amino acids are present: Tyr, Trp, Met, iLeu, Thr, Val, Leu, and Lys, and optionally Arg and His, but no other amino acids;

(2) Lys is present in an amount of from 5 to 30 mg per 500 mg of total LNAA supplement

(3) the supplement is substantially free from Phe, and

(4) the weight ratio of Leu to iLeu is greater than 1:2

Applicant respectfully submits that the Examiner has not fully appreciated the core of the presently claimed invention, which is not simply a result of finding the optimum percentages of lysine in an "already generally known" supplement. The presently claimed invention is entirely different from Wachtel. Though Wachtel is directed to a product to be used by PKU patients, Wachtel does not teach or suggest that PKU patients could be <u>treated</u> in order to control the

uptake of Phe from the normal diet, much less that the treatment could (or would) be improved by blocking the uptake of Phe from the GI tract (from the intake of other foods), or how that could be accomplished.

There is no teaching in Wachtel of using increased amounts of amino acids which compete with Phe for GI tract uptake and uptake over the blood brain barrier (BBB). Thus, the reasoning behind the present invention is not taught by Wachtel. As Wachtel does not mention or suggest blocking the uptake of Phe over the GI tract (from the intake of other foods), a person of skill in the art would have had no reason to try to modify Wachtel to arrive at the present invention.

As discussed previously, Applicant submits that the technology of Pietz et al., which is described in the present specification beginning on page 28 ("Pietz") should be considered as more pertinent prior art than Wachtel. Pietz's work formed the theoretical basis for the commercial product Prekunil®, with which supplement mixtures according to the presently claimed invention were compared and found surprisingly better. Pietz is concerned with the same problem as the present invention (blocking Phe uptake into the brain) as the present invention.

Pietz relates to the testing of LNAA supplements and how they block Phe transport into the brain in patients with PKU. The aim of the study in Pietz was to use the approach to further investigate Phe transport through the BBB in the patients by manipulating blood concentrations of Phe as well as other LNAA's e.g. by giving an LNAA mixture comprising Val, Met, iLeu, Leu, Tyr, His and Trp. With supplement of LNAA's, Pietz observed that Phe influx into the brain was completely blocked. The research studies led to the development of Prekunil®, the commercial LNAA supplement for treatment of PKU preceding the present invention. Applicant has attached as Exhibit A a brochure describing Prekunil®, entitled "Prekunil® Enjoy Full Liberty of Choice."

Nothing in Exhibit A, which discloses the background for and content of the competitive LNAA supplement for reducing uptake of Phe over the BBB, or in Pietz, which was what spurred the development of Prekunil®, suggests that the treatment it describes could or would be improved by blocking the uptake of Phe over the GI tract in addition to blocking the uptake over the BBB. Nor does it provide any guidance on how to accomplish this.

That the presently claimed invention would not have been obvious over Wachtel, Prekunil, or Pietz, is further demonstrated by additional experimental results.

For example, Table 6 of the present specification shows data for SuppM2, which is a supplement according to present claim 1. Administration of SuppM2 to two mice, P433 and P482, resulted in a 27% reduction in Phe plasma level, whereas the reduction with Prekunil was 18% and in the control group 13%. The Examiner has contended that the study reflected in the specification, particularly the data in Table 6, does not demonstrate unexpected results relative to the compositions of Pietz or Wachtel. Applicant disagrees with the Examiner's position, and offers the following additional experimental evidence in support of the patentability of the presently claimed invention.

Applicant has conducted further trials both in mice and in patients from different PKU treatment centers. The results are disclosed in two articles published in 2006 (attached as Exhibit B) and 2007 (attached as Exhibit C) in the Journal of Inherited Metabolic Disease (JIMD), one of the most important scientific journals for the field of PKU research, and in three abstracts from the same journal (attached as Exhibits

D, E, and F) . These trials were conducted with a composition according to the claims, NeoPhe (see table 1 of Exhibit B) which is slightly different from the composition of SuppM2 disclosed in the specification, but still with the scope of the present claims.

A number of experimental trials were conducted. A first trial was a pilot project conducted over a week, and is described in Exhibit D. Fourteen patients with PKU were given 0.5 g/kg of NeoPhe per day in three divided doses taken with meals. After two days of treatment, the mean blood Phe level was decreased from 1266 µmol/L to 1073 µmol/L. After 1 week the mean blood Phe level was decreased to 869 µmol/L, corresponding to a mean decline of 32%. Attached as Exhibit G is a set of the slides used in a lecture given in Paris in September 2005 describing this study.

A second trial is described in Exhibit E. This trial was a short-term, double blind placebo control trial conducted over a month. Sixteen patients from different PKU centers participated. They were given 0.5 g/kg/day of either NeoPhe (a composition according to the invention) or a placebo in three divided doses taken with meals. A significant blood Phe drop was seen in patients receiving NeoPhe, averaging 27% from baseline.

During the placebo treatment blood Phe did not vary significantly. The author concluded that the study indicates that LNAA can compete with Phe on the transporter in the GI tract.

A third trial was a long term study (reflected in Exhibit F), in which 4 patients were given 0.5 g/kg/day of NeoPhe in three divided doses to be taken at mealtime. The patients were not on medical food for more than 10 years before the trial, which was reflected in high blood levels of Phe at the start of the trial (a mean value of 1507 µmol/L). The patients' blood Phe levels were determined after 2 weeks and once a month for a period of 12 months. The mean blood Phe level declined for each of the subjects during the period to 642 µmol/L, 707 µmol/L, 899 µmol/L and 869 µmol/L. These levels are within NIH recommendations. There were no complaints from the patients, and they asked to continue taking NeoPhe.

Other trials are described in Exhibits B and C. These trials show a decline of 52% in blood Phe concentration in 8 patients, starting from a baseline of blood Phe of 957 µmol/L, taking 0.5 g/kg per day of a LNAA supplement of the invention and a decline of 55% in 3 patients, staring from a baseline of 1230 µmol/L, taking 1.0 g/kg per day. Both these results

represent significant declines of blood Phe levels. Similar declines were seen in tests on mice test (see Exhibit B, page 3, under "Results". The results are also reflected in Figure 1-3 in the article.)

Exhibit B also explains the theory behind the treatment, viz. the competition of LNAA's in the GI tract which occurs by increasing the concentration of LNAA's and Lys (see, page 5, column 1, last paragraph, to column 2, third paragraph.)

The results of the study on Exhibit B are further supported by the study described in Exhibit C. That study was a double blind placebo control trial of large neutral amino acids in treatment of PKU. Twenty patients from 6 different PKU centers were treated with 0.5 g/kg per day NeoPhe, and again a significant decline in Phe plasma concentration was observed. For patients already on a PKU formula were also seen a drop in Phe plasma concentration (see page 155 and Figure 1.)

The foregoing experimental data as a whole clearly demonstrates the excellent and surprising results achieved using the compositions according to the presently claimed invention

In summary, none of the prior art teaches or suggests that altering the amount of Lys in Wachtel's composition would result in the excellent treatment results discussed in the

specification and in the attached articles. Therefore, Applicant respectfully submits that the § 103(a) rejection should be reconsidered and withdrawn.

Conclusion

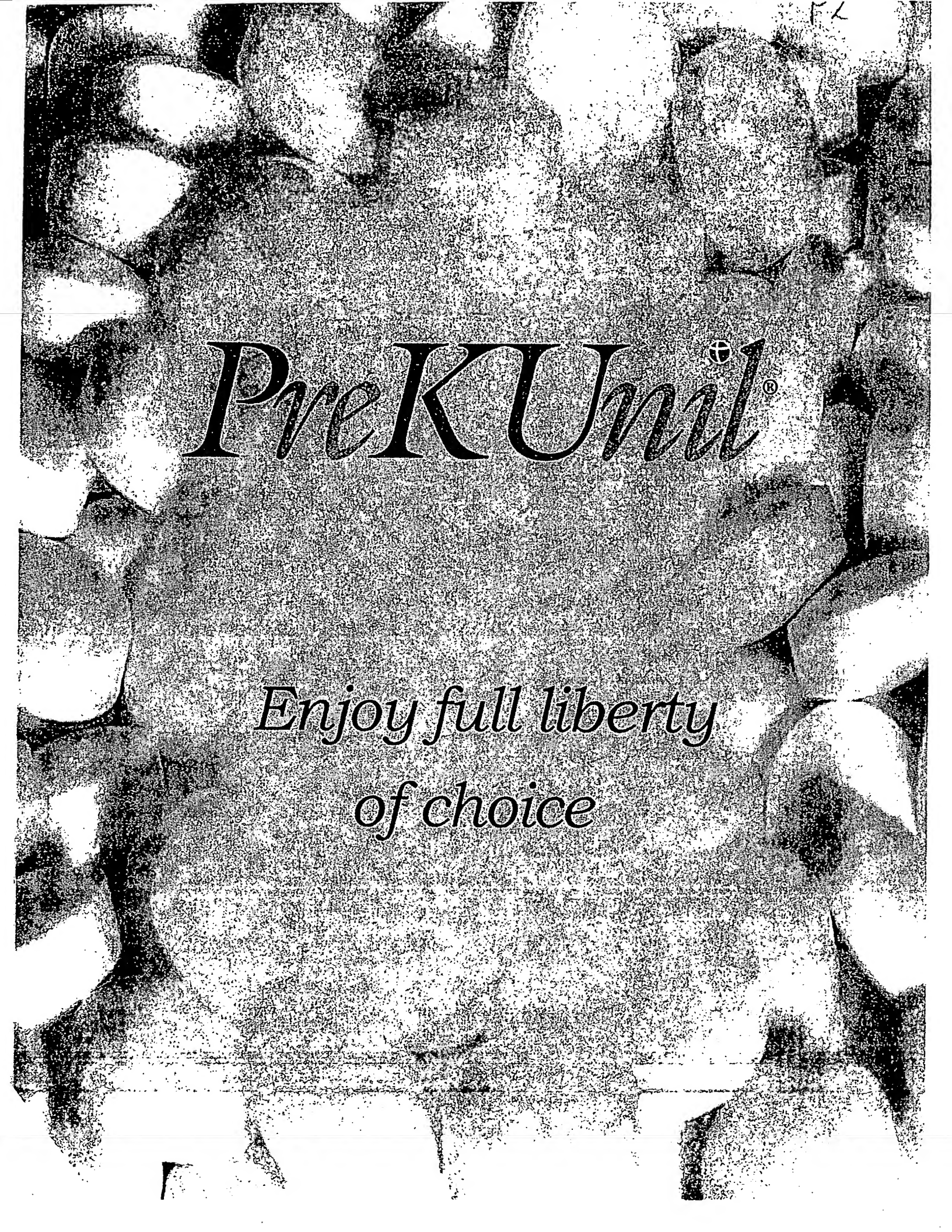
Applicant believes the currently pending claims are now in condition for allowance. If the Examiner has any questions regarding this response, the Examiner is invited to telephone Applicant's counsel at the number provided below.

Respectfully submitted,
JACOBSON HOLMAN PLLC

By _____
Harvey Jacobson, Jr.
Reg. No. 20,851

400 Seventh St., N.W.
Washington, D.C. 20004
(202) 638-6666

EXHIBIT A


PreKUnit®
Enjoy full liberty
of choice

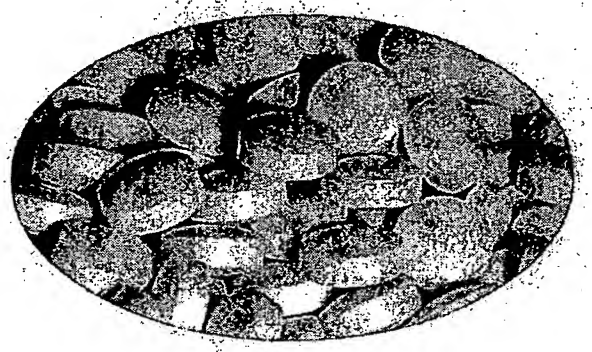
Facts about PKU

Phenylketonuria (PKU) is an inherited inborn error of metabolism, if untreated affecting the central nervous system and causing irreversible mental retardation. The disease is caused by the total lack or substantially reduced effect of the liver enzyme phenylalaninehydroxylase. This leads to an accumulation of the amino acid phenylalanine in the blood, 10-20 times the normal level. The high inflow of phenylalanine from the blood is toxic to the brain and disturbs the normal developement of brain- and nerve-function.The lack of phenylalaninehydroxylase is caused by a genetic defect or mutation. Healthy parents of a PKU child are carriers of only one mutation. To get the disease you must have a double set of the mutation, The statistical risk of two persons carrying a PKU mutation giving birth to a child with the disease is one to four, i.e 25%.

In 1934, the Norwegian physician Asbjörn Fölling demonstrated the connection between symptoms of PKU and impaired metabolism of phenylalanine. In the 1950's it was proven that a special protein restricted diet could reduce the toxic levels of this amino acid.

PKU is caused by a variation of hundreds of different mutations, which results in different stages of the disease. Patients with HPA (hyperphenylalanimia) still have some phenylalaninehydroxylase activity and less elevated phenylalanine blood levels and do not usually need dietary treatment.
For best results, the proteinrestricted diet should be initiated as early as the first month of life. In order to do so, many countries screen every newborn child by taking a blood test within the first week of birth.

Over the decades, scientists have debated the age at which the PKU patient can return to a normal diet. Nowadays most specialists are of the opinion the treatment should continue during adolescence and early adulthood. Some even consider lifelong treatment to be preferred. Rescent research shows that patients, who have returned to a normal diet too early, suffer from a slightly impaired brainfunction and intellectual performance (attention and working memory).



The traditional dietary treatment of classical PKU

Treatment of phenylketonuria is monitored by a low phenylalanine diet, and by regular estimations of blood phenylalanine concentrations.

The dietary restriction of natural protein required to control blood phenylalanine levels, compared to the protein required for growth and mental development and the correction of the imbalance of serum amino acids necessitates the use of a protein substitute low in phenylalanine to supplement the natural protein intake.

This means that proteinrich food like eggs, meat, fish, cheese, lentils and beans are totally avoided and other articles of foods which are relatively high in protein are permitted in very small quantities such as ordinary bread, rice, potatoes, and pasta. To get the required energy for the age the diet has to be supplemented with formulated low protein bread, low protein pasta, low protein milk and more.
As the tolerated levels of phenylalanine intake differs, the diet should be individually adapted.

The necessity of a well controlled dietary treatment

Thirty years of experience of early (< 3 weeks postnatal) wellcontrolled dietary treatment (blood phenylalanine levels 2-5 times normal) for the first 10-15 years of a person's life have revealed normal cognitive development (IQ), normal neuropsychological testperformances and usually a normal psychiatric status and social adjustment (Azen et al.1991; Smith 1994; Weglage et al.1996; Griffiths et al.1998). Studies of persons treated in early childhood with good dietary control suggest that the risk of intellectual deterioration declines dramatically after the first decade: the inference being that from 10 years of age and onwards the nervous system may be sufficiently mature to withstand the neurotoxic influence of persistent hyperphenylalaninemia on IQ (Smith et al. 1990; Smith 1994; Beasley et al. 1994; Schmith et al.1996; Griffiths et al.1998)

The risks involved in discontinuance of dietary treatment

A review of 21 published articles on neuropsychological performance in adolescency and young adults off diet who early were treated fairly strictly, revealed deficits in abstract reasoning, both in conceptual and visuospatial areas.

Some PKU individuals displayed deficits when required to integrate information (Waisbren et al.1994). Studies of young adults off diet by Pennington et al.1985 and Welsh et al.1990 suggested that higher levels of problemsolving, i e "executive function" were most noticeably affected. Individuals with PKU were not significantly different from controls on simple reaction time tests (tests of visual motor speed), but they tended to make more errors and to slow down to a greater extent as the complexity of the task increased (reviewed by Waisbren et al.1994. In addition, they showed greater variation in their response time, indicating a deficit in sustained attention (Sonneville et al. 1990; Schmith et al. 1992; Weglage et al.1996).

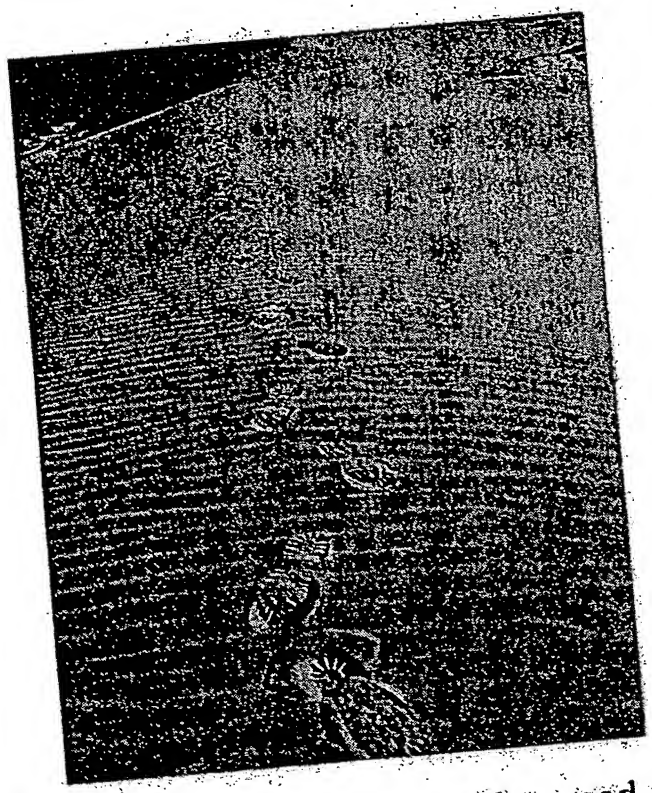
In addition to neuropsychological effects, it has been demonstrated that as phenylalanine concentration increases, the water content of white matter increases, an effect, which becomes visible on magnetic resonance imaging (MRI) when concentration increases above 600 µmol (Bick et al. 1991; Lou et al.1992; Thompson et al.1993).

In addition to MRI findings, there have been reports of soft neurological signs (tremor, unusually brisk tendon reflexes) in more than 30% of early treated wellcontrolled young adults who have been on a normal diet for many years (Krause et al.1985; Clarke et al.1987; Endres 1998; Cerone et al.1998).

The above data would appear to favour the view that the risk of deteriorated brain function and integrity due to higher blood phenylalanine levels persists into late childhood and probably throughout life, even though the risks in adulthood are less (Smith 1994).

Advantages contra disadvantages of continual treatment

The advanteges of continual treatment with PKU are that the treatment reduces key biochemical abnormalities like impaired amino acid transport across the blood-brain barrier, reduces the risk of impaired dopamine and serotonin synthesis, reduces the risk of a probable impaired myelin turnover, and that treatment improves neuro-psychological performances and may improve MRI changes (Smith 1994).
The disadvantages of treating adults with PKU are that it is a difficult regime, it needs constant monitoring and support, there are risks of nutrient deficiency or imbalance and even that the gain is uncertain and disputed.



The choice of treatment regime

Confronted with all these advantages and disadvantages in continual treatment in adulthood and probably throughout life, it is understandable that young people with PKU are faced with a very difficult decision whether to continue or to stop treatment.

PKU centres worldwide are having difficulties justifying going off the diet, except for patients with mild PKU with levels below 700 µm/l on a normal diet.

However, it can be very difficult to motivate PKU patients to continue with a low protein diet and drinking formula throughout life. Indeed whatever final conclusion emerges regarding the risks of giving up the diet. Each patients view is likely to have an increasingly important place in forming an opinion in every instance.

The need for amino acids

Records on young adults with PKU who decided to give up a low-phenylalanine diet often reveal that the young adults have put themselves on a highly protein restricted diet, i.e. 0,5 g per kilo bodyweight in order to avoid headache and symptoms like hangover.

Such a highly protein-restricted diet is dangerous because all the main components of the body consist of protein constructed from amino acids; essential amino acids are particularly important.

The importance of tyrosine and tryptophan

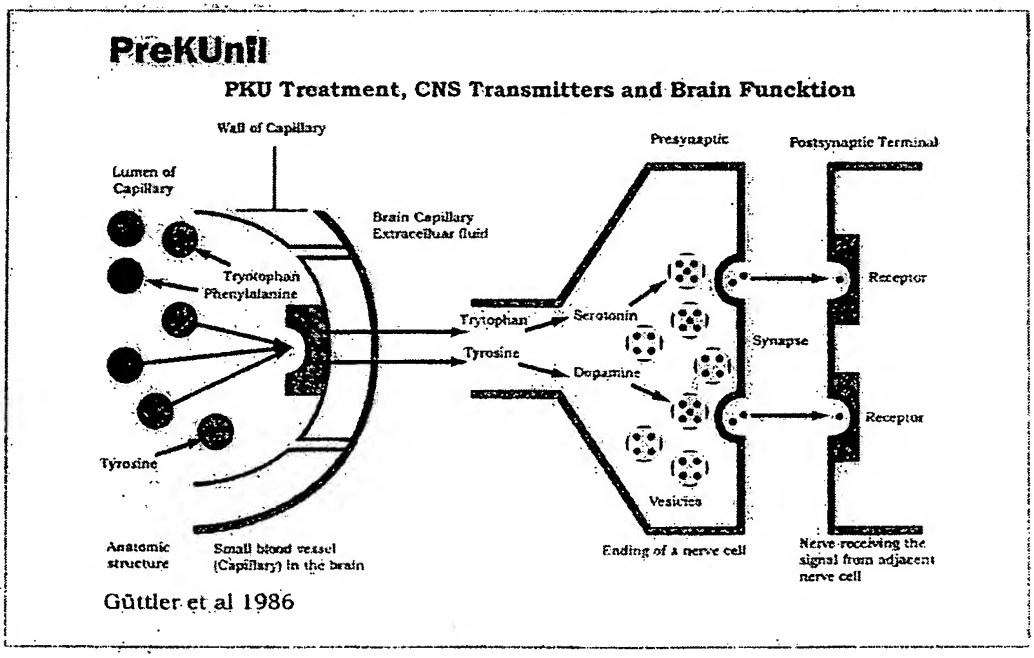
For individuals with PKU tyrosine is a very important essential amino acid since these individuals can not form tyrosine from phenylalanine. Tyrosine is the precursor of dopamine, noradrenaline and adrenalin. In addition, tyrosine is the precursor of the thyroid hormone, thyroxin. The concentrations in the cerebrospinal fluid of the neurotransmitters, dopamine and serotonin, are reduced in untreated hyperphenylalaninemia. (Butler et al.1981; Lou et al.1985; Villasana et al.1989; Thompson et al.1990)

Possible mechanisms for the impaired neurotransmitter synthesis include decreased transport of precursor amino acids across the blood-brain barrier into the neuronal cell and inhibition of phenylalanine and tryptophan hydroxylase (Güttler and Lou 1986).

Deficiencies in these neurotransmitters seem to play a role in behavioral disturbances associated with untreated PKU (Baumeister et al.1983; Lewis and Baumeister 1982; Ernst et al.,1996; Schroeder et al.1997; Baumeister and Baumeister 1998).

Moreover, dietary supplementation with tyrosine and tryptophan in untreated PKU has been reported to increase serotonin and dopamine synthesis and improve performance on behavioral tests (Lou 1985; Lou et al.1987; Lykkelund et al.1990).

Tyrosine and Tryptophan are competing for the same receptor in the blood brain barrier with Phenylalanine (Phe). High dosages of tyrosine and tryptophan will in theory block the absorption of phenylalanine across the blood-brain barrier. The main problem having PKU is intoxication of the brain due to high levels of Phenylalanine.



When amino acids (AA) diffuse through the wall of blood vessels in the brain (the blood brain barrier) into the cells, the phenylalanine, tyrosine and tryptophan compete for the same bonding sites.

High levels of tyrosine and tryptophan in the blood reduce the uptake of phenylalanine by simple competition. As more tyrosine and tryptophan reaches the nerve cells, further important neurotransmitters are produced. Tyrosine is transformed into dopamine and nor adrenaline and tryptophan is transformed into serotonin. These transmitters are stored in small vesicles until released and thus transfer the nerve signal to the receptors and the adjacent nerve cell. (Güttler et al.1986)

The effects of the branched chain amino acids

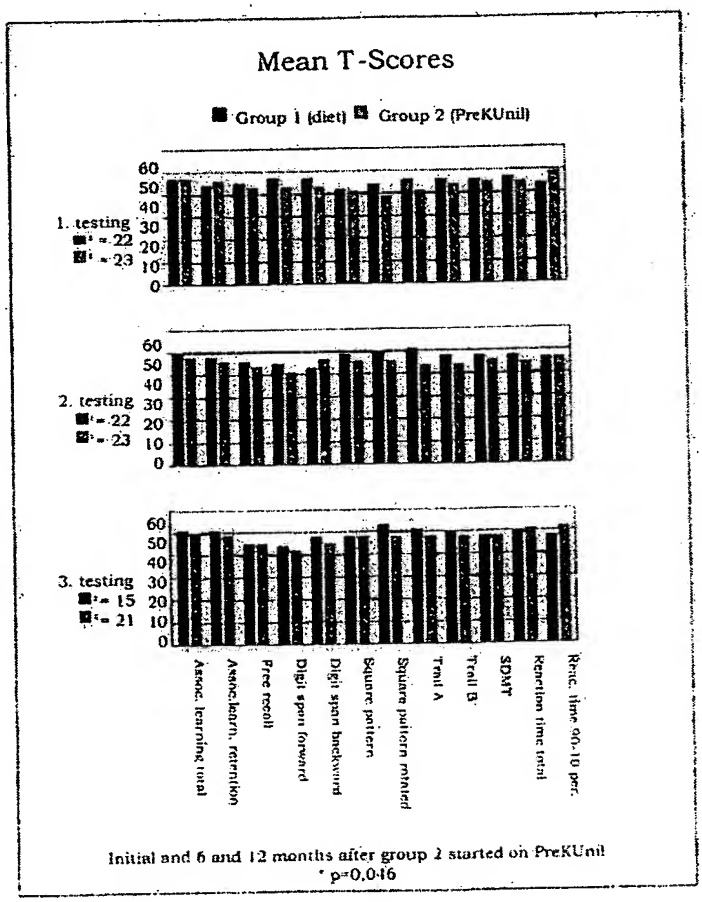
The neutral amino acids (valine, isoleucine, leucine, tyrosine, tryptophan and phenylalanine) are transported across the blood-brain barrier by a common carrier (Patridge 1983).

By increasing the concentrations of the amino acids that compete with phenylalanine for transport, it may be possible to lower brain phenylalanine and thus to reduce any neurotoxic effect.

In fact, the branched chain amino acids have been reported to reduce phenylalanine concentrations in the cerebrospinal fluid in PKU patiens (Anderson and Avins 1976; Berry et al. 1977; Berry et al.1982).

Furthermore, a number of studies have found that a diet supplemented with branched chain and other neutral amino acids produce an improvement in neurological, cognitive and behavioural measures (Jordan et al.1985; Crowley et al.1990; Berry et al.1990).

Conclusion:
In conclusion, these studies provide evidence for reversibility of certain biochemical and behavioural effects in hyperphenylalaninemia following diet discontinuance when a proteinrestricted free diet is supplemented with amino acids (PreKUnil-tablets), vitamins, minerals and trace elements (cf Baumeister and Baumeister 1998).



The psychological test battery as shown in columns 1-12 assesses different Neuropsychological parameters such as attention, vigilance and visuomotor functions, as well as learning and retention. No statistical differences were found in patients on PreKUnil tablets compared with patients on a normal PKU diet. These tests were performed after 12 months of PreKUnil treatment.

PreKUnil®

tablets

- Are developed by the Danish company Nilab and John F. Kennedy institute in collaboration. The John F. Kennedy institute is responsible for national (nation wide) PKU treatment in Denmark, Faroe Island and Greenland.
- Are based on systematic scientific studies and on experimental results (Andreasen J et al., 2001, Aaring K.K et al. 1999) indicating that PreKUnil may neutralise the negative effect of giving up a low-phenylalanine diet. Thus, a semi-free diet limited in protein and supplemented with vitamins, minerals and trace elements should be safe, provided that the adult with PKU supplements the semi-free diet with amino acids contained in PreKUnil tablets.
- Have been used in Denmark since 1985 to treat several hundreds of PKU patients
- Are used worldwide in more than 12 countries
- Are an amino acid (AA) supplement, including all essential AA with a large dosage of two selected AA: Tyrosine and Tryptophan.

Clinical trials with semi-free diet supplemented with PreKUnil show neuropsychological test results to be as good as when the more restricted diet is applied (figure on page 6)

A pilot study conducted at Children Hospital Los Angeles using MRS (Magnetic resonance spectroscopi) to evaluate brain Phe concentrations compared to blood Phe concentrations using LNAA therapy

Large Neutral Amino Acid therapy and Phenylketonuria: A Promising Approach to Treatment

Richard Koch,[a] M.D. Kathryn D. Moseley,[a] M.S.,RD, Shoji Yano,[a] M.D., Marvin Nelson, Jr.,[b] M.D., Rex A Moats. Ph.D.[b]

[a]Division of Medical Genetics and Radiology Department MS #90, Childrens Hospital of Los Angeles
[b]Department of Pediatrics, Keck School of Medicine University of Southern California.

Abstract:

Six subjects with classical phenylketonuria were treated with large neutral amino acid supplements (PreKUnil, PreKUlab) at 0,4 tablets/kg/day in equally divided doses three times each day on a increased natural protein diet. All six subjects had low or deficient blood concentrations of both tyrosine and tryptophan, which are precursors for dopamine and serotonin, respectively, at the beginning of the study and were increased substantially throughout the study. Blood phenylalanine concentrations remained essentially unchanged, while the brain phenylalanine concentrations gradually decreased toward the carrier range as seen in parents of children with PKU. Two subjects were diagnosed with clinical depression and were in counseling programs at initiation of the study. At the end of the study all patients reported increased energy and overall improvement in well-being.

Methods:

Six individuels (4 females and 2 males) ranging in age 20-34 years agreed to participate in this study which was approved by the investigation review board and informed consent was obtained from each participant according to hospital policy. At baseline 24h. 48h, one month and six mounths, each subject received an MRS and plasma amino acids were obtained. Diet records were obtained and analyzed at each visit. Immediately after baseline measurements were obtained each subject was given PreKUnil tablets (PreKUlab) at 0,4 tablets/kg body weight/day. The average dose was 10 tablets to be taken before each meal (3 times each day). They were instructed to consume a "relaxed" diet, which included unlimited fruits, vegetables, and grains, with small amounts of cheese and yougurt and up to two small servings per week of meat, if desired. Vitamin/mineral tablets were added as well as calcium supplements.

The MRS procedures have been well described. Mutation analyses of phenylalanine hydroxylase gene were performed at the John F. Kennedy Institute in Glostrup, Dk. Plasma amino acids were obtained using a Hitachi 8800 amino acid analyser. Diet records were analyzed using th Amino Acid Analyzer, version 3.3 produced by Ross Metabolics,

Resluts:

Tabele 1 details the subjects' profile. All were classifeid as classical PKU with servere mutations.The diet records were collected and analyzed at each visit and indicate their average protein intake ranged from 0,6-1,0 g/kg each day.

The mean blood Phe value for all six subjects at baseline was 1,448 mM (24 mg /dl). Subsequent means at 14 and 48 h after starting oral PreKUnil tablets at the recommended dose of 0,4 t. /kg/day were 1.393 and 1,453 mM,(23 mg/dl, and 24 mg/dl), res-pectively. The mean levels at one month and six months of therapy were slightly lower at 1.345 and 1.315 mM (22 mg/dl) (see Fig.1). Blood tyrosine (Tyr) and tryptophan revealed a different pattern of response. Over the period of treatment a gradual increase of tyrosine from the baseline value of 0,033 (0,5 mg/dl) to 0.081 mM (1,3 mg/dl)(Fig, 2) and tryptophan from 0.030 (0,72 mg/dl) to 0.073 mM (1,8 mg/dl)(Fig. 3). In contrasting the blood data to the brain Phe concentrations, results over the six months of treatment resulted in a decrease from 0.452 mM at baseline to 0.265 mM (Fig. 4). All subjects repoted having more energy while on the PreKUnil and felt better overall. One subject was lost to follow up after three months in the study due to unknown reasons.Two subjects who were depressed also clinically improved.

Figure 1

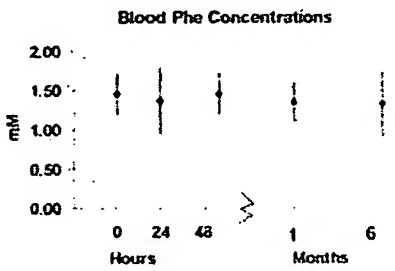


Figure 2

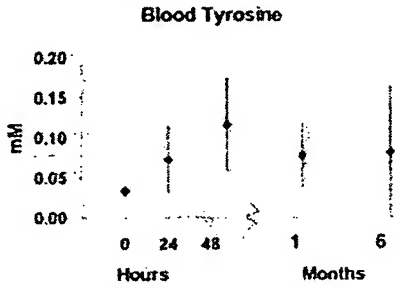


Figure 3

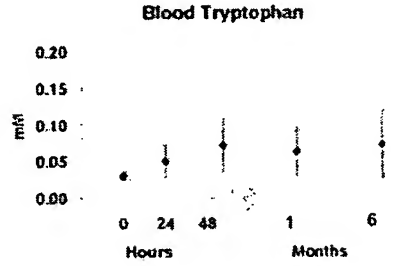


Figure 4

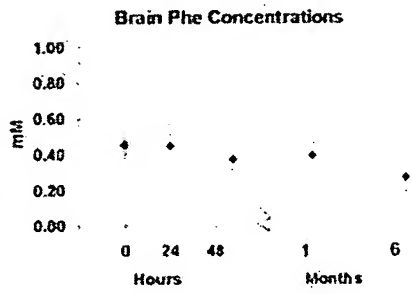


Table 1
Subject profile

Subject	Age	Sex	Diet	Mutation	Weight	Protein intake (g/kg)	Phe int. (mg/kg)
1	29	F	Y	R408/R408W	65	0.8	38
2	34	M	Y	Y387H/delT323	78	1.0	43
3	34	F	N	R158Q/IVS10nt-11	90	0.7	32
4	20	M	Y	IVS12nt1g-a/165T	70	0.8	33
5	33	F	N	L348V/L348V	48	1.0	43
6	24	F	N	IVS12nt/165T	84	0.6	23

Subject 5 and 6 off diet >10 years Protein intake includes protein equivalent from PreKUnil

Discussion:

For over 40 years the focus of treatment for persons with PKU has been on blood phenylalanine control. At this institution we have performed MRI/MRS studies on over a hundred PKU individuals to determine brain Phe concentrations compared to blood Phe concentrations. We have data on 5 normal controls and 4 carriers (parents of PKU individuals), Based on our observations, we believe that brain Phe concentration can be used as a measure to determine appropriate blood Phe concentration on an individual basis.

In this study three of the four females participating have been off diet for over 10 years with blood Phe concentrations well over 1.2 mM (20 mg/dl). Since PreKUnil is not recommended for pregnant females due to the increased blood Phe concentrations above the recommended range for pregnancy, all participants were taking birth control measures.

Subjects 1 and 2 had always taken the prescribed medical product but blood Phe concentrations were typically above 1.2 mM (20 mg/dl). It is interesting to note that the blood Phe concentrations overall were reduced slightly despite an increased protein and the brain Phe concentrtions showed a clear reduction over the six months with wide variability. This trend is consistent with Dr, Reuben Matalon's (Children's Hospital, Galveston, TX) experiments in the PKU mice that were fed supplements made from PreKUnil tablets.

Two persons observed with mild depression were both involved in counseling programs. Both had made significant clinical improvement by the end of the study. In all six subjects blood concentrations of tyrosine and tryptophan were in the low range or deficient despite the increased protein intake at baseline. Also, the ratio of Phe/Tyr was decreased on average from 44.5 to 15.1.

In two subjects leucine and isoleucine were in the deficient ranges prior to PreKUnil therapy. Both were normalized after 48 h. of treatment. It is highly suggestive that the depression that we see in PKU is related to diminished dopamine and serotonin levels in the brain caused by the low blood concentrations of tyrosine and tryptophan. However, the brain Phe concentrations also decreased as well as the ratio of brain to blood Phe, which may be a contributing factor.

Based on the Danish experience and in these preliminary findings, we suggest that balancing the ratios of amino acids in blood and the subsequent effect this has on the level of those amino acids in the brain is of relevance in maintaining intellectual achievement and neurological health. The impact of this novel approach to treatment also has significance for treating the depression, which often accompanies persons with PKU off dietary treatment. No adverse clinical symptoms occurred due to the approach to treatment. Clinically, all six subjects reported improvement of general demeanor and increased energy despite similar blood Phe concentrations maintained throughout the treatment period.The two subjects who were mildly depressed at the initiation of treatment reported overall improvement as well. The occurrence of depression is a clinically serious disorder and was the basis for offering them the opportunity to participate in this study. Future studies are needed to evaluate and quantify these psychological findings on a larger scale.

In view of the fact that many adults and adolescents are either off diet or do not adhere to the Phe-restricted diet, it would be prudent to offer the LNAA therapy. The use of the LNAA therapy has been shown to improve amino acid profiles as well as increase tyrosine and tryptophan concentrations in the blood,

which are precursors of dopamine and serotonin. Our findings indicate that the brain concentration of phenylalanine decreases toward the carrier range within six months of therapy with PreKUnil tablets despite increased natural protein intake, In addition, the product was well accepted by all six subjects.

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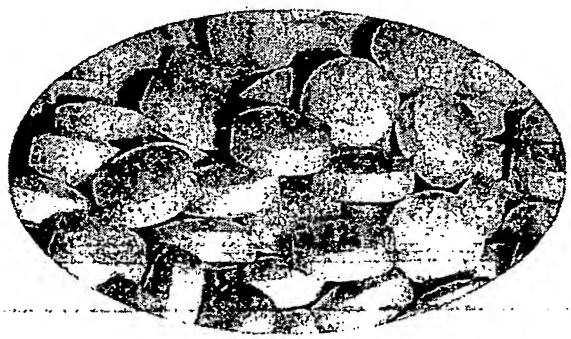


PreKUnil®

tablets

- Are intended for patients on PKU diet treatment from about the age of 12 and onwards. For treatment of younger patients a doctor specialising in PKU should be consulted
- Are not recommended for maternal PKU's, where low phenylalanine is crucial for successful outcome of pregnancy
- Are not to intended for use as a sole source of nutrition
- Must be supplemented with a moderate restricted PKU diet (semi free) and multi-vitamin/mineral tablets
- Have to be taken in three or more dosages distributed over the day along with a meal. The blocking effect of tyrosine and tryptophan preventing phenylalanine from entering the brain does only work if food and PreKUnil are consumed at the same time, which makes it crucial to take PreKUnil along with a meal
- Are swallowed with water like other tablets
- Are without any smell or taste

Ingredients:
256 mg
L-Tyrosine, L-Tryptophan
244 mg
L-Methionine, L-Isoleucine, L-Threonine, L-Valine, L-Leucine, L-Histidine, L-Arginine
Inulin
Constituents:
E 342, E 553b, E 572, E 551, E 904, E 460

Nutritional information:	
100 g tablets	
Energy	1050 kJ
	250 kcal
Protein	0 g
Amino acids	62.5 g
Carbohydrate	0 g
Fat	0 g
Does not include any vitamins or minerals	

PreKUnil tablets make the difference

PKU patients on a low protein diet only get 20% of their protein requirement from natural food and the rest 80% from amino acid powder or tablets.
This is necessary in order to keep the level in the blood and brain within the recommended range.

Using PreKUnil tablets the main source of protein 80% comes from natural protein in food and only 20% comes from PreKUnil tablets. The patient is able to eat a semi free and much more relaxed diet. The higher intake of phenylalanine from natural protein is allowed due to the fact that phenylalanine will only enter the blood stream but not the brain because of the blocking effect of high dosage of tyrosine and tryptophan.

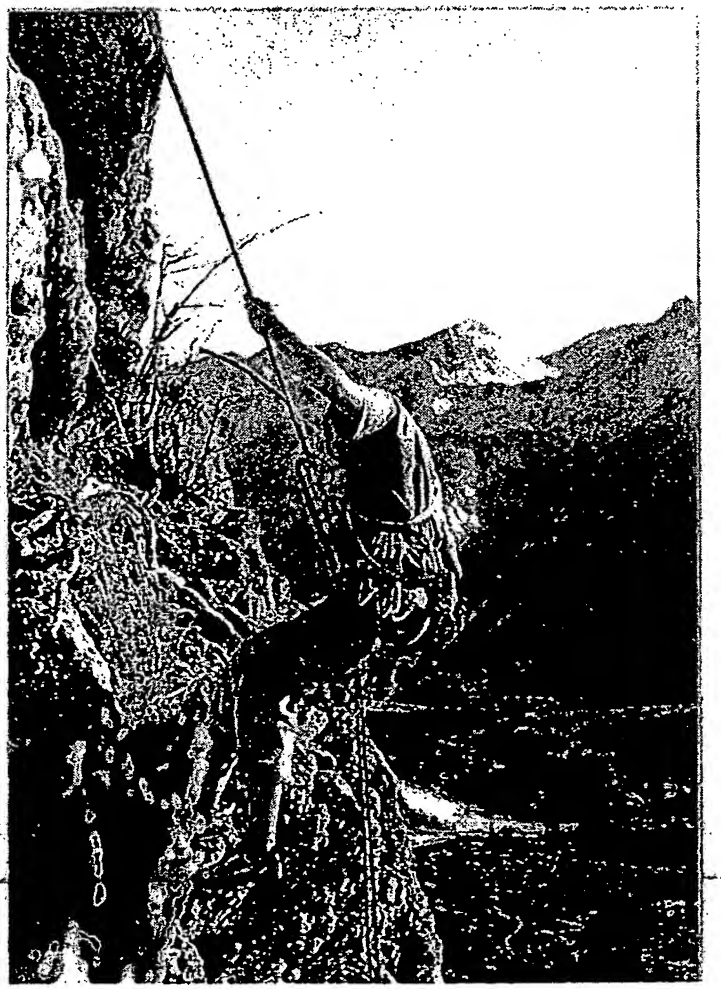
The semi free diet includes normal bread, rice, pasta, vegetables, fruit, fatty fish and meat. The intake of fish, egg, dairy products such as cheese, milk, yoghurt and lean meat should be discussed or avoided.

The number of tablets a PKU-patient needs is determined according to the body weight.

Body x 0,4 = number of tablets

The tablets cover 20% of the protein requirement per day. The rest 80% comes from natural protein. It is important to eat sufficient amount of natural protein from normal food. Recommended protein intake is 1 g protein/kg/day.

Example:
Male 20 years 75 kg
75 x 0,4 = 30 tablets
(10 t. 3 times a day along with a meal)



A typical Danish menue with supplements as PreKUnil or amino acid formula

	PreKUnil & Semi free diet	Amino acid powder and low protein diet
Breakfast:	Normal bread, honey, marmalade, butter, normal cereals, PKU-milk, 10 PreKUnil tablets	Low protein bread, honey, marmalade, low protein cereals, PKU-milk 50 g of formula
Snack:	Fruit	Fruit
Lunch:	Normal bread, bread spread (fat, meat), butter, vegetables, 10 PreKUnil tablets	Low protein bread, vegetables marmalade, exchanges* 50 g of formula
Snack:	Fruit	Fruit
Dinner:	Unlimited amounts of rice, pasta or potatoes, 50 g of fat meat, vegetables, 50 ml of cream sauce, 10 PreKUnil tablets	Limited amounts of potatoes, rice or pasta, low protein pasta, low protein rice, vegetables, 50 ml of cream sauce 50 g of formula
Snack	Fruit	Fruit
Protein from food	60 g	15 g
Amino acids	15 g	60 g
Phe, total	3000 mg	750 mg

* exchanges are equael to 50 mg of phenylalanine

PreKUnil®

Ideal for:

- Young adults from the age of 15, who have been diagnosed from birth and well treated with LP diet and AA powder trough childhood and teenage.
- Late diagnosed and untreated PKU patients, who will have difficulties coping with a low protein diet and drinking formula
- Short periods of time for younger patients with PKU, where coping with low protein diet and drinking formula can be difficult, like on hiking trips, school camps etc.

Since PreKUnil tablets are a dietery supplement, consisting AA in a specific combination, it is considered harmless and without any side effects. Too high amounts ot tyrosine are known to cause headache.

After almost 20 years of experience from Denmark without any reports of side effect, the product has been approved in other countries like the US, Germany, Poland, Turkey and Sweden.

PKU patients can always choose to go back on LP diet and AA powder or tablets. PreKUnil is not addictive, but should be considered as a necessary, dietary supplement for PKU patients who choose to live on a semi free diet.

PreKUnil®

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beneholm@worldonline.dk

EXHIBIT B

Exhibit B

D 11

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ORIGINAL ARTICLE

Large neutral amino acids in the treatment of phenylketonuria (PKU)

R. Matalon · K. Michals-Matalon · G. Bhatia · E. Grechanina · P. Novikov · J. D. McDonald · J. Grady · S. K. Tyring · F. Guttler

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Summary Large neutral amino acids (LNAAs) have been used on a limited number of patients with phenylketonuria (PKU) with the purpose of decreasing the influx of phenylalanine (Phe) to the brain. In earlier studies on mice with PKU (ENU^2/ENU^2), LNAAs were given and a surprising decline in blood Phe concentrations was observed. The formula used in the mouse experiment (PreKUnil) lacked lysine. Therefore, a new formulation of LNAAs (NeoPhe) was developed, introducing changes in the concentration of some amino acids and adding lysine, so that such a mixture could be used in humans. The new formula was found to be effective in reducing blood Phe concentration in mice by about 50% of the elevated levels. Patients with PKU were given LNAAs and blood Phe concentrations were determined in an open-label study. Three centres—in Russia, the Ukraine and the USA—took part in the study. NeoPhe was given at 0.5 g/kg per day in three divided doses to eight subjects with PKU and at 1.0 g/kg per day to three patients, for one week. The NeoPhe resulted in decrease of elevated blood Phe by 50% in both groups. The preliminary data from this study are encouraging and a double blind placebo-controlled trial will be required to show long-term efficacy and tolerance of LNAAs in the treatment of PKU.

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Competing interests: None declared

R. Matalon (✉) · G. Bhatia · J. Grady
Department of Pediatrics, University of Texas Medical Branch, Children's Hospital, Galveston, Texas, USA
e-mail: rmatalon@utmb.edu

K. Michals-Matalon
University of Houston, Houston, Texas, USA

E. Grechanina
Institute of Clinical Genetics, Kharkiv State Medical University, Kharkiv, Ukraine

P. Novikov
Department of Clinical Genetics, Institute of Pediatrics and Child Surgery, Moscow, Russia

J. D. McDonald
Wichita State University, Wichita, Kansas, USA

S. K. Tyring
University of Texas-Health Science Center, Houston, Texas, USA

F. Guttler
Kennedy Institute, Glostrup, Denmark

Abbreviations

CSF	cerebrospinal fluid
5-HIAA	5-hydroxyindolacetic acid
LNAA	large neutral amino acid
Phe	phenylalanine
PKU	phenylketonuria
VIL	valine, isoleucine and leucine

Introduction

Phenylalanine-restricted diet for the treatment of PKU started following the early trials of Bickel and colleagues (1953) about 50 years ago. Experience gained from treating PKU showed the efficacy of dietary treatment in preventing mental retardation, even with some diet relaxation as children got older. Recently, 'Diet for life' has been advocated beyond childhood years (Azen et al 1991; Fisch et al 1997; Gleason et al 1992; Holtzman et al 1986; Michals et al 1985; Walters et al 2002). When blood Phe concentrations are not brought down to what is considered a therapeutic concentration, problems with poor school performance, impaired executive

functioning, changes in the white matter of the brain and loss of intelligence quotient may be encountered (Burgard et al 1997; Diamond 2001; Fisch et al 1995; Griffiths et al 1995; Lou et al 1985; Michals et al 1988; Pietz et al 1998; Ris et al 1994; Schmidt et al 1994; Scriver and Kaufman 2001; Seashore et al 1985; Smith et al 1978, 1991; Thompson et al 1990, 1994; Walters et al 2002). Therefore, in order to prevent blood concentrations of Phe exceeding a certain acceptable concentration, alternative modes of therapy for PKU are being sought, so that some reduction of blood Phe concentrations continues throughout the life of patients with PKU (Scriver and Kaufman 2001).

Clinics have adopted their own criteria for acceptable concentration of blood Phe for adults with PKU. In order to have uniformity in the treatment of PKU and to have a range of acceptable blood Phe concentrations in the USA, the National Institutes of Health (NIH) convened a consensus conference and issued guidelines for treating PKU. Blood Phe concentration of 120–360 μmol/dl were recommended for young children 0–13 years; for those of 13 years and older the concentration are allowed to go higher, 900 μmol/dl (NIH 2001). In Europe the guidelines for adults are different and blood Phe concentrations of 1200 μmol/dl are acceptable (MRC 1993).

The first approach to lowering blood Phe concentrations and compliance with dietary treatment was attempted by companies who make formulas for PKU. In order to improve treatment, PKU formulas were made more palatable and were offered in a variety of flavours and textures such as gels, bars, tablets and other forms. The new formulas are more acceptable to adults, but still compliance has been limited.

A recent addition to therapy is the use of (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) for treatment of PKU. Kure and colleagues (1999) reported that patients with mild PKU responded to BH_4 by significant reduction in their blood Phe. Subsequent studies (Blau and Scriver 1997; Blau and Trefz 2002; Erlandsen et al 2004; Kure et al 1999; Lassker et al 2002; Lindner et al 2003a, b; Matalon et al 2003, 2004; Muntau et al 2002; Spaapen et al 2000; Trefz et al 2000, 2001; Weglage et al 2002) confirmed the findings of Kure and colleagues. It seems that patients with mild missense mutations in one allele may show significant decline in their blood Phe concentrations when given BH_4. From our experience with BH_4, about 50% of patients may benefit from BH_4 but only about 10% of patients will require BH_4 as a monotherapy and the other 40% of the patients who respond to BH_4 will require dietary restriction of Phe in addition to BH_4 treatment (Matalon et al 2002, 2004).

Interest in the possibility that large neutral amino acids (LNAAs) could lower brain Phe was initiated by the work of Oldendorf and colleagues (Oldendorf and Szabo 1976). In order to cross the blood–brain barrier, amino acids require a transporter protein. The large neutral amino acids and the cationic amino acids (phenylalanine, tyrosine, tryptophan, threonine, isoleucine, leucine, valine, methionine, lysine, arginine and histidine) share a common transporter to the brain and compete with one another (Choi and Pardridge 1986; Hargreaves and Pardridge 1988; Pardridge 1977, 1982; Pardridge and Oldendorf 1975). Pardridge (1982) showed that the transport of LNAAs on the carrier protein and the movement of amino acids across the blood-brain barrier depend on the affinity of each amino acid for the carrier protein. So far, studies with PKU have concentrated on the blood-brain barrier and the reduction of entry of Phe into the brain compartment.

Large neutral amino acids and cationic amino acids cross the intestinal mucosa by means of a carrier protein similarly their crossing of the blood–brain barrier, except that the affinity of the amino acids for the intestinal carrier is determined by a K_m two orders of magnitude higher than that of the blood-brain carrier. In this study we document the lowering of blood Phe concentration in humans and mice with PKU using a mixture of LNAAs supplied in the diet, suggesting that competition at the intestinal carrier protein can be achieved.

Materials and methods

The mixture of LNAAs (NeoPhe) was obtained from Prekulab, Korsor, Denmark. The composition of NeoPhe is shown in Table 1 and is compared to PreKUnil, which is not suitable for long-term use in humans because of potential lysine deficiency if it is taken with limited protein intake. In NeoPhe, lysine and histidine are added and leucine is increased. Mice with PKU ($n = 7$), genotype ENU^2/ENU^2 (classical PKU), were purchased for the University of Texas Medical Branch (UTMB) laboratory from Jackson Laboratories, Bar Harbor, ME, USA. The ENU^2 mutant mouse strain was produced at the University of Wisconsin by exposure

Table 1 Comparison of PreKUnil and NeoPhe[a] (LNAA) composition per tablet

L-Amino acid	PreKUnil (mg)	NeoPhe (mg)
Tyrosine	128	195
Tryptophan	128	51
Methionine	35	32
Isoleucine	35	35
Threonine	35	32
Valine	35	35
Leucine	35	130
Histidine	0	30
Lysine	0	30
Arginine	35	30
Total	466	600

[a]Prekulab, Denmark

of founder animals to *N*-ethyl-*N'*-nitrosourea, which caused a F263S point mutation, resulting in classical PKU in homozygous animals. Mice used in this study were genotyped to verify that they were homozygous for the F263S mutation according to the method of McDonald and Charlton (1997). The group of mice at UTMB, Galveston and at Wichita State University were given 16.7% NeoPhe blended into their normal chow diet. This was done following ICAUC approval. On average, mice eat approximately 6 g of chow daily. The addition is 1 g of LNAAs to 5 g of chow, which contains about 50 mg of Phe, so the ratio of LNAAs to Phe is very high. Blood Phe was determined four times in one week before NeoPhe and twice in one week while on NeoPhe, and twice following cassation of treatment.

Patients with PKU were recruited from three centres: the Institute of Clinical Genetics, Kharkiv State Medical University, Kharkiv, Ukraine; the Department of Clinical Genetics, Institute of Pediatrics and Child Surgery, Moscow, Russia; and the Department of Pediatrics, University of Texas Medical Branch, Children's Hospital, Galveston, Texas, USA. The patients enrolled in the open-label study had to have PKU and be old enough to swallow pills. Each patient signed an institutionally approved informed consent prior to enrolment and all the patients were genotyped earlier.

Patients in the Ukraine and Russia were treated with Tetraflex early in life. Tetraflex is similar to other formulas in Europe. The patients in the study were not in optimal dietary control, and Phe intake exceeded 500 mg/day. The patients from the US clinic were older and were on 'vegetarian' diet; their Phe intake exceeded 1000 mg/day.

There were 11 patients, 4 male and 7 females. Eight patients (mean age 20.5 years) received 0.5 g/kg per day and 3 patients (mean age 16.5 years) received 1.0 g/kg per day of NeoPhe divided into three doses and taken before meals. Patients were instructed to continue their diet as they did prior to enrolling in the trial. Baseline Phe was determined on four separate occasions and at zero time and post NeoPhe at one week. Blood Phe was also determined one week after NeoPhe treatment. Blood Phe was assayed using filter paper and tandem mass spectroscopy (Pediatrix, Bridgeville, PA, USA).

Paired *t*-tests were used to assess changes from baseline measurements with the T-Test procedure using SAS statistical software (*SAS/STAT 9.1 User's Guide*; SAS Institute, Inc., Cary, NC, USA).

Results

The mean blood Phe concentration for mice ($n = 7$) was determined four times during nine days on normal chow. The average concentrations were used as a baseline for each mouse. The average Phe concentration of two blood samples while on NeoPhe was used for post-LNAA testing. Blood Phe concentrations were decreased by 53%, from an average of 1444 to an average of 678 μmol/dl. This decline in blood Phe is statistically significant ($p < 0.0001$) as shown in Fig. 1. The identity of each mouse is shown on the right of the graph. Blood Phe decreased on average by 766 μmol/dl (SD = 197). Since mice could not manipulate their intake and constantly ate the same diet, the decrease in their blood Phe concentrations clearly shows 'proof of principle' that LNAAs in NeoPhe can reduce blood Phe concentrations.

Blood Phe concentration in the 8 patients (Fig. 2) taking 0.5 g/kg per day of LNAAs decreased from an average of 957.4 μmol/dl to an average of 458.4 μmol/dl, a decline of 52%, which is statistically significant ($p = 0.004$). There were three patients (Fig. 3) who took 1 g/kg per day of LNAAs. Their baseline average was 1230 μmol/dl compared to an average of 549.0 μmol/dl, an average decline of 55%.

All patients experienced a decrease in blood Phe concentrations from baseline after NeoPhe. The average decrease was 601 μmol/dl (SD = 370), and when analysed together ($N = 11$) this drop in blood Phe was highly significant ($p = 0.0003$).

When treatment was discontinued, blood Phe concentrations increased to pre-trial levels.

Discussion

Lack of adherence to diet in the treatment of PKU has resulted in neuropsychological deficits, even with early detection and treatment (Burgard et al 1997; Diamond 2001; Fisch et al 1995; Griffiths et al 1995; Lou et al 1985; Michals et al 1988; Pietz et al 1998; Smith et al 1978; Thompson et al 1990). This created the need to reassess treatment strategies and prompted the National Institutes of Health (NIH) in the USA to convene a consensus conference so that recommendations for treatment guidelines could be reached (NIH 2001). The difficulty in attaining the goal of blood Phe of 120–600 μmol/dl in adolescents was highlighted at the conference, and was further documented by the report of Walters and colleagues (2002). There have been ongoing attempts to find other modalities for therapy of PKU. A promising method for lowering blood Phe concentrations in patients with mild PKU is supplementation with BH_4 (Blau and Trefz 2002; Kure et al 1999; Lindner et al 2003a, b; Matalon et al 2002, 2004; Muntau et al 2002; Trefz et al 2000, 2001; Weglage et al 2002). Studies with BH_4 indicate that approximately 50% of patients with PKU have a positive response to BH_4. Clinical trials of long-term treatment with BH_4 are now in progress.

The idea of utilizing the competition of LNAAs with Phe on the carrier protein for the blood–brain barrier has been entertained for some time. The transport of LNAAs to the brain is mediated by a carrier protein with the lowest K_m

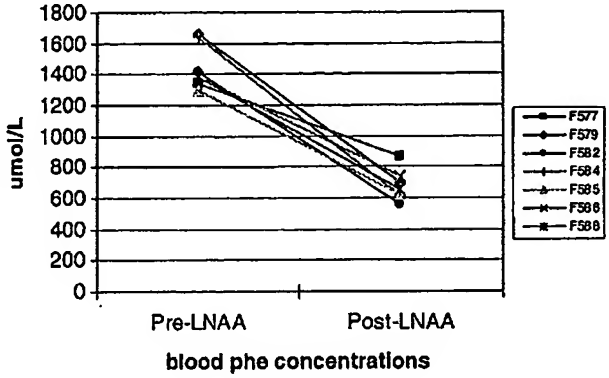

Fig. 1 Mean blood Phe concentration with standard deviation of ENU^2/ENU^2 mice (n=7) on normal chow (pre-LNAA) and on 16.7% NeoPhe. The ID of each mouse is given at the right. The reduction in blood Phe level is 53% from baseline with p-value <0.0001

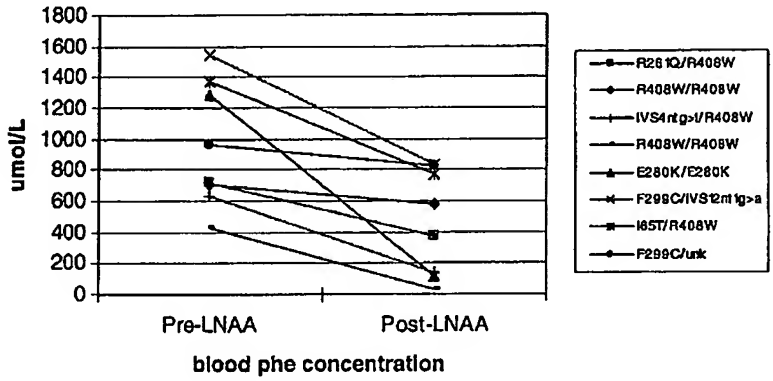

Fig. 2 Blood Phe response to 0.5 g/kg NeoPhe in 8 patients with PKU, showing 52% average decline in blood Phe concentration

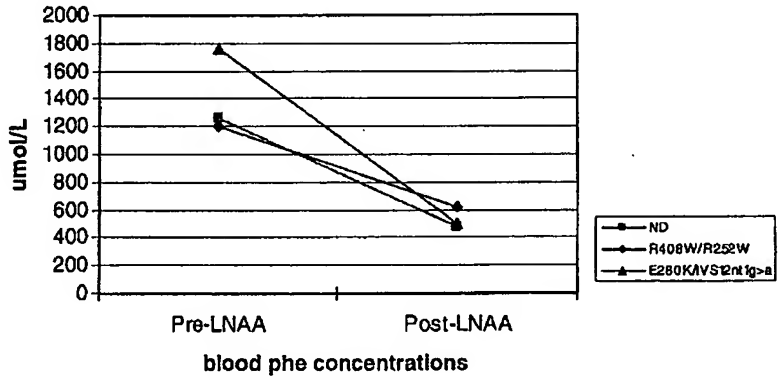

Fig. 3 Blood Phe response to 1.0 g/kg NeoPhe in 3 patients with PKU, showing 55% average decline in blood Phe concentrations

for Phe. The Michaelis–Menten constant (K_m) for each large neutral amino acid is dictated by the equation:

$$K_m(\text{app}) = K_m\left(1+\sum[\text{aa}]/K_m^{\text{aa}}\right) \quad (1)$$

The K_m apparent (app) of a given amino acid deviates from the absolute K_m in the presence of a competing amino acid. The K_m equation predicts that if the plasma concentration of an LNAA is much less than its value of K_m, then that amino acid will not compete effectively for the carrier protein. In PKU, blood Phe is much higher than other LNAAs so Phe can displace other amino acids and readily cross the blood–brain barrier (Pardridge 1982). Therefore, Phe is favoured to cross the blood–brain barrier rather than other LNAA (Choi and Pardridge 1986; Hargreaves and Pardridge 1988; Moller et al 1997; Oldendorf and Szabo 1976). The lower concentrations of tyrosine, tryptophan and branched-chain amino acids that have been reported in patients and mice

with PKU (Smith and Kang 2000) are the result of the competition of Phe with other LNAAs to cross the blood–brain barrier because of its higher concentrations in blood.

Clinical trials with tyrosine on patients with PKU started with the work of Lou and colleagues (1985), who gave 160 mg/kg of tyrosine to patients and reported increased attention span and neurotransmitter synthesis, as judged by neurotransmitter metabolites in the CSF. Subsequent studies by Pietz and colleagues (1995) giving 100 mg/kg tyrosine/kg to 24 early-treated PKU patients for 4 weeks showed no beneficial effects in neuropsychological tests. The addition of tryptophan to the diet of patients with PKU resulted in increase in 5-HIAA in CSF, while Phe concentration were unaltered. No effect on neuropsychological performance or vigilance was observed (Neilsen 1987). Berry and colleagues (1982, 1990) used branched-chain amino acids to inhibit the influx of Phe to the brain. Valine 150 mg/kg, isoleucine 150 mg/kg, and leucine 200 mg/kg (VIL) were used in these studies. The patients on VIL had a substantial lowering of Phe in the CSF, but tyrosine concentrations also were lower. Since VIL is not a complete mixture of LNAAs and does not contain tyrosine and tryptophan, which are precursors for neurotransmitters, it is not considered an adequate mixture of amino acids for the treatment of PKU (Hommes 1989).

The first study of LNAA supplementation in the treatment of PKU was conducted using formulas of LNAAs without lysine, such as PreKUnil (Dotremont et al 1995). Four patients were treated for one month using a formula with 0.8 g/kg LNAAs and a low-protein diet, 0.6 g/kg. The treatment led to negative nitrogen balance due to lysine deficiency, indicating that such a formulation was not adequate for treatment of PKU.

A different study by Pietz and colleagues (1996) used Phe loading in six male patients with PKU, who were given Phe 100 mg/kg with and without LNAAs. When treated with LNAAs, the influx of Phe to the brain was decreased as measured by magnetic resonance spectroscopy (MRS). Moats and colleagues (1999, 2000), using MRS to measure brain Phe, studied patients on 0.6 g/kg of LNAAs and showed a decrease in brain Phe concentration following the treatment with LNAAs. The use of MRS to measure brain Phe concentration is technically difficult and it is not an accepted method for routine follow-up on treatment in PKU. A method that relies on blood determination of Phe concentration would be the preferred method, since this is an accepted practice.

In the GI tract, LNAAs are also transported by a carrier protein with a K_m that is two orders of magnitude higher than that of the CNS carrier protein. Lysine and arginine are also transported on the same carrier protein (Hidalgo and Borchardt 1990; Larsen et al 1964; Pardridge 1982). According to the experiments of Hidalgo and Borchardt (1990), using human intestinal-epithelial cells (Caco-2-cells in monolayers with a buffer containing 10 μmol/L Phe), significant inhibition of Phe transport requires 100-fold (1 mmol/L) LNAAs, as dictated by the K_m equation for affinity of LNAAs to the GI carrier protein. For example, at such concentrations, leucine inhibits Phe transport by 55%, tyrosine by 45% and the cationic amino acid lysine by 50%.

Under physiological conditions, competition of LNAAs with Phe is not likely to occur in the GI tract. However, by increasing the concentration of LNAAs and lysine while Phe is unchanged or reduced, competition with the GI transporter can be achieved. When tested on mice with PKU mouse chow with 16.7% LNAAs, a statistically significant decrease in blood Phe concentration was observed. This suggests that a competition with the transport of Phe can be attained with high concentrations of LNAAs in the GI tract, satisfying the concentrations required by the K_m for the GI transporter. Earlier, studies with PreKUnil resulted in lowering of blood and brain Phe in mice (Matalon et al 2003.)

The same mixture of LNAAs (NeoPhe) was used in patients with PKU, and also resulted in lower blood Phe concentration in the treated patients, suggesting that the mixture leads to increased concentrations of LNAAs in the GI tract competition with Phe transport.

The data presented indicate that the inhibition of transport of Phe can occur in the GI tract using LNAAs at the concentrations used in the study. It is also possible that better utilization of amino acids and protein synthesis is increased (anabolic effect). In mice we have not seen weight gain. In patients the trial was too short to document anabolic effects. Since this is a preliminary study, future experiments should focus on the mechanism of lowering of blood Phe concentration. The decline in blood Phe in PKU patients taking LNAAs is easy to measure and it is being reported for the first time. It is possible that if natural protein is somewhat limited (less Phe), more effective competition of LNAAs with Phe should be further enhanced, leading to lower blood Phe concentrations.

The 11 patients with PKU in this report were mostly classical PKU patients, as seen by the blood concentration of Phe and their genotype. Only two patients in this group responded to BH_4, while the others had no decline in blood Phe concentrations following loading with BH_4. None of these patients was on BH_4 during the study. These findings indicate that LNAAs can be effective in reducing blood Phe in all PKU patients. It is possible that patients who respond partially to BH_4 and still require Phe restriction will benefit from combination of BH_4 and LNAAs.

Strategies similar to the lowering of blood Phe concentration with LNAAs can be developed for the treatment of the tyrosinaemias. In this case Phe and tyrosine (and methionine in some cases) can be excluded from the LNAA preparation. Maple syrup urine disease (MSUD) can be treated similarly by exclusion of leucine, isoleucine and valine, limiting their absorption from the GI tract. Limiting methionine

in homocysteinuria while increasing the other LNAAs may prove helpful in lowering blood homocysteine concentrations. Similarly, leucine absorption can be decreased by increasing the composition of other LNAAs in the GI tract. All such treatments need to be carefully planned and monitored in specialized metabolic centres, with the goal that diet in such diseases can be relaxed so that better compliance is achieved and metabolic crises are less frequent.

The data from this study suggest that double-blind placebo-controlled clinical trials should be conducted with LNAAs. Trials with similar formulas should be conducted in specialized centres, with the understanding that these pills are only a supplement and not a complete diet. Energy, micronutrients and other essential nutrients should be provided by the daily diet.

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EXHIBIT C

Exhibit C D 12

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Double blind placebo control trial of large neutral amino acids in treatment of PKU: Effect on blood phenylalanine

R. Matalon · K. Michals-Matalon · G. Bhatia · A. B. Burlina · A. P. Burlina · C. Braga · L. Fiori · M. Giovannini · E. Grechanina · P. Novikov · J. Grady · S. K. Tyring · F. Guttler

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Summary Large neutral amino acids (LNAA) have been used on a limited number of patients with phenylketonuria (PKU) with the purpose of decreasing the influx of phenylalanine (Phe) to the brain. In an open-label study using LNAA, a surprising decline of blood Phe concentration was found in patients with PKU in metabolic treatment centres in Russia, the Ukraine, and the United States. To validate the data obtained from this trial, a short-term double-blind placebo control study was done using LNAA in patients with PKU, with the participation of three additional metabolic centres – Milan, Padua and Rio de Janeiro. The results of the short trial showed significant lowering of blood Phe concentration by an average of 39% from baseline. The data from the double-blind placebo control are encouraging, establishing proof of principle of the role of orally administered LNAA in lowering blood Phe concentrations in patients with PKU. Long-term studies will be needed to validate the acceptability, efficacy and safety of such treatment.

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References to electronic databases: Phenylketonuria (PKU; OMIM 261600)

R. Matalon (✉) · G. Bhatia · J. Grady
Department of Pediatrics, University of Texas Medical Branch, Children's Hospital, Galveston, Texas, USA
e-mail: rmatalon@utmb.edu

K. Michals-Matalon
University of Houston, Houston, Texas, USA

A. B. Burlina · A. P. Burlina
Inherited Metabolic Disease Unit and Department of Neuroscience, Neurological Clinic, University Hospital of Padua, Italy

C. Braga
Diagnósticos Laboratoriais Especializados and Centro Ambulatorial de Prevenção/APAE-Rio de Janeiro, Brazil

L. Fiori · M. Giovannini
Department of Pediatrics, San Paolo Hospital, University of Milan, Italy

E. Grechanina
Institute of Clinical Genetics, Kharkiv State Medical University, Kharkiv, Ukraine

P. Novikov
Department of Clinical Genetics, Institute of Pediatrics and Child Surgery, Moscow, Russia

S. K. Tyring
University of Texas-Health Science Centre, Houston, Texas, USA

F. Guttler
Kennedy Institute, Glostrup, Denmark

Abbreviations

BBB	blood–brain barrier
LNAA	large neutral amino acids
Phe	phenylalanine
PKU	phenylketonuria
VIL	valine, isoleucine and leucine

Introduction

Phenylketonuria (PKU) is caused by deficient activity of the enzyme phenylalanine hydroxylase (PAH) (Folling 1934; Jervis 1953; Kaufman 1971). The treatment of PKU with diet restricted in phenylalanine (Phe) has become a standard care following the early trials of Bickel and colleagues (1953).

Experience with the treatment of PKU indicated efficacy of the low-Phe diet with the possibility of diet relaxation in older children. Studies showing decline in intellectual

performance when blood Phe concentrations were high resulted in reassessment of the policy of diet relaxation. Gradually the concept of 'Diet for Life' emerged on the basis of subsequent studies (Azen et al 1991; Fisch et al 1997; Gleason et al 1992; Michals et al 1985; Walter et al 2002). Decline of intellectual performance when blood Phe concentrations are elevated is the basis for continued diet in PKU. When blood Phe concentrations are high, individuals with PKU often have problems with poor school performance, decline in executive functioning, and changes in white matter of the brain. (Burgard et al 1997; Diamond 2001; Fisch et al 1995; Griffiths et al 1995; Lou et al 1985; Michals et al 1988; Pietz et al 1998; Ris et al 1994; Schmidt et al 1994; Smith et al 1978, 1991; Scriver and Kaufman 2001; Seashore et al 1985; Thompson et al 1990, 1994). In order to prevent blood Phe from exceeding acceptable concentrations, different modes of therapy have been advocated (Scriver and Kaufman 2001).

Centres that treat PKU have advocated different blood Phe concentrations for young children or adults, so that uniformity of acceptable Phe concentration has been lacking. A consensus conference organized by NIH (NIH 2001) with experts from the United States, the United Kingdom, Germany, France and other countries resulted in guidelines suggesting blood Phe concentrations of 120–360 μmol/L for children from birth to 13 years of age. Those who are 13 years and older are recommended to have blood Phe concentration not exceeding 900 μmol/L, with concentration below 600 μmol/L preferred. In Europe, in some centres, blood Phe concentration of 1200 μmol/L is allowed. In the UK, specific guidelines were developed (MRC Working Party on Phenylketonuria 1993), although some centres in the UK accept 1200 μmol/L. Even with these higher blood Phe recommendations it is still difficult to attain desired blood Phe concentrations (NIH Consensus Report on Phenylketonuria 2001).

The discovery that (6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) could reduce blood Phe in some patients with PKU (Kure et al 1999) was met with enthusiasm. Subsequent studies (Blau and Scriver 1997; Blaue and Trefz 2002; Erlandsen et al 2004; Lassker et al 2002; Lindner et al 2003a,b; Matalon et al 2003, 2004; Muntau et al 2002; Spaapen et al 2000; Trefz et al 2000, 2001; Weglage et al 2002) confirmed the findings of Kure and colleagues. However, the response to BH_4 is primarily limited to patients with mild PKU.

Large neutral amino acids (LNAA) have been suggested for use in treatment of PKU because of the competition with Phe at the blood–brain barrier (BBB). Oldendorf and Szabo (1976) showed that LNAA cross the BBB with the same transporter protein that is also shared by cationic amino acids. The large neutral amino acids and the cationic amino acids (phenylalanine, tyrosine, tryptophan, threonine, isoleucine, leucine, valine, methionine, lysine, arginine histidine and other cationic amino acids) share a common transporter to the brain and compete with one another (Choi and Pardridge 1986; Hargreaves and Pardridge 1988; Hidalgo and Borchardt 1990; Pardridge 1977, 1982; Pardridge and Oldendorf 1975). Pardridge (1982) showed that the transport of LNAA and movement of amino acids across the BBB depend on the affinity of each amino acid for the carrier protein.

Large neutral amino acids and cationic amino acids cross the intestinal mucosa by a carrier protein similar to that of BBB, except that the affinity of the amino acid for the intestinal carrier has a K_m two orders of magnitude higher than that of the BBB, so that under physiological conditions high concentrations of LNAA need to be present in the GI tract in order to compete with Phe. Recently, we have shown that blood Phe concentration in patients with PKU, as well as in mice with PKU, decline significantly when treated with LNAA (Matalon et al 2006).

We report the results of a study using LNAA in patients with PKU carried out by several metabolic centres in different countries. The data from open-label studies showing decline of blood Phe concentration in patients with PKU were encouraging and indicated that a mixture of LNAA (NeoPhe) can successfully lower blood Phe concentrations in patients with PKU (Matalon et al 2006). This double-blind study confirms previous findings that LNAA can be used for all patients with PKU who need reduction in blood Phe concentration.

Patients and methods

Tablets of large neutral amino acids (NeoPhe) were obtained from Prekulab, Korsor, Denmark. The composition of LNAA is the same as described in the open-label study (Matalon et al 2006). The placebo tablets were supplied by the same company and were identical in size and appearance. The placebo contained lactose monohydrate, microcrystalline cellulose and colloidal hydrated silica.

Patients with PKU were recruited from six centres: Department of Pediatrics, University of Texas Medical Branch, Children's Hospital, Galveston, Texas, USA; Institute of Clinical Genetics, Kharkiv State Medical University, Kharkiv, Ukraine; Department of Clinical Genetics, Institute of Pediatrics and Child Surgery, Moscow, Russia; University of Milan, Italy; Inherited Metabolic Disease Unit and Department of Neuroscience, Neurological Clinic, University Hospital of Padua, Italy; Diagnósticos Laboratoriais Especializados and Centro Ambulatorial de Prevenção/APAE- Rio de Janeiro, Brazil. The patients enrolled in the study had to have PKU and be old enough to swallow pills. Each patient signed an institutionally approved informed consent prior to enrollment.

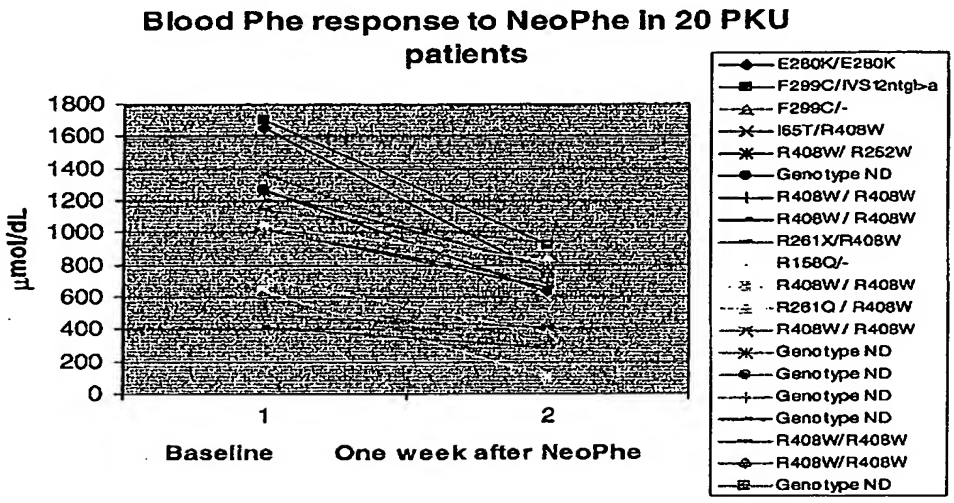

Fig. 1 Blood Phe response to NeoPhe in 20 PKU patients after one week. Average blood Phe concentration at baseline was 932.9 µmol/L, which dropped to 568.4 µmol/L (average drop of 364.5, SD = 232.2, $p < 0.0001$). Seven patients with mean baseline of 531.6 µmol/L, which dropped to 281.5 µmol/L (average drop of 250.1, SD = 73.7, $p = 0.009$)

There were 20 patients in the study, aged 11 to 32 years, 12 female and 8 male. There were 4 patients from the USA, 5 from Ukraine, 1 from Milan, 2 from Padua, 2 from Brazil, and 6 from Russia. With the exception of one patient, the 20 patients had classical PKU as indicated by the clinical classification based on initial Phe concentration and genotype.

The patients were instructed to continue their diet as prior to enrolling in the trial. The dosage of LNAA was as 0.5 g/kg per day in three divided doses to be taken with meals, which is about one tablet per kg per day. The placebo tablets were given in the same dosage. This dosage was acceptable and patients complied with the treatment. The order of the placebo and experimental treatments was random and was unknown to the patient and the treating physician. Compliance and randomization were supervised by the metabolic centres. Baseline Phe was determined on three separate occasions prior to active participation in the study. The baseline is the point of comparison of the patient's current treatment and the double-blind study. Pills containing either placebo or NeoPhe were administered and blood Phe was determined twice weekly. Each centre took blood at the same time for each visit, usually two hours after meals. The patients had a one-week washout period prior to the next week of the double-blind crossover trial. Blood Phe was again assayed at the beginning and twice weekly during the second phase.

Paired *t*-tests were used to assess changes from baseline measurements with the *t*-test procedure using SAS statistical software (SAS Institute, 2004).

Results

At the end of the double-blind trial the results were unmasked. Blood Phe concentration in the 20 patients from all the participating centres dropped significantly while on 0.5 g/kg per day of LNAA (NeoPhe). The average blood Phe concentration at baseline, taken on three separate occasions, was 932.9 µmol/L. During the week of NeoPhe (LNAA), the average blood Phe concentration dropped to 568.4 µmol/L (average drop of 364.5, SD = 233.2), a decline of 39%, which was highly statistically significant ($p < 0.0001$) as indicated in Fig. 1. Seven patients with classical PKU who adhered to treatment with their PKU formula also showed a drop in blood Phe concentration from baseline of 531.6 µmol/L, which dropped to 281.5 µmol/L (average drop of 250.1, SD = 173.7, $p = 0.009$) when NeoPhe was given (Fig. 1).

The results of the placebo trial showed minor changes in blood Phe concentrations when compared to baseline levels. The average blood Phe concentration changed from 932.9 µmol/L to 882.66 µmol/L, a decline of 5.4%, which was not statistically significant ($p = 0.07$), as indicated in Fig. 2.

Discussion

In a recent report of a one-week open-label trial with LNAA, blood Phe concentrations decreased without any change in the dietary practice of the patients with PKU (Matalon et al 2006). These results were encouraging and seemed to raise the possibility of a new modality for the treatment of PKU. As patients with PKU grow older, their dietary adherence erodes, and this is associated with neuropsychological deficits (Burgard et al 1997; Diamond 2001; Fisch et al 1995; Griffiths et al 1995; Lou et al 1985; Michals et al 1988; Pietz et al 1998; Smith et al 1978; Thompson et al 1990). According to the NIH conference (NIH 2001), the blood Phe concentration should be 120–600 µmol/L in adolescents, a

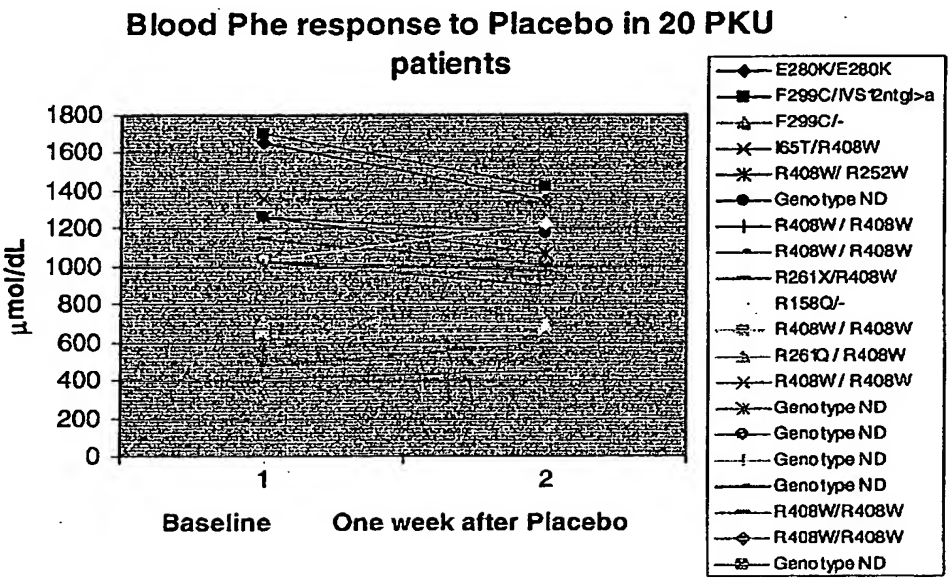

Fig. 2 Blood Phe response to placebo in 20 PKU patients after one week of washout. Average blood Phe concentration at baseline was 932.9 µmol/L, which dropped to 882.6 µmol/L, a decline of 5.4% (not statistically significant)

level difficult to attain in older children and adults (Walter et al 2002). Tetrahydrobiopterin (BH_4) can lower blood Phe in some patients; however, most patients will still require dietary Phe restriction and only mild PKU patients will benefit from BH_4 as the sole method of treatment (Blau and Trefz 2002; Kure et al 1999; Lindner et al 2003a,b; Matalon et al 2002, 2004; Muntau et al 2002; Trefz et al 2000, 2001; Weglage et al 2002).

In the past, trials with LNAA in the treatment with PKU have focused on the transport of Phe to the brain. Trials with tyrosine, 160 mg/kg, to patients with PKU showed increased attention span and neurotransmitter synthesis, as judged by neurotransmitter metabolites in the CSF (Lou et al 1985). However, Pietz and colleagues (1995) gave 100 mg/kg tyrosine to 24 early-treated PKU patients for four weeks and showed no improvement in neuropsychological tests.

Studies using valine 150 mg/kg, isoleucine 150 mg/kg, and leucine 200 mg/kg (VIL) resulted in substantial lowering of Phe in the CSF, but tyrosine was also lowered. The first study of LNAA supplementation in the treatment of PKU was conducted using a formula of LNAA without lysine, such as PreKUnil (Dotremont et al 1995). Four patients were treated for one month using a formula with 0.8 g/kg LNAA and a low-protein diet, 0.6 g/kg. The treatment led to negative nitrogen balance due to lysine deficiency, indicating that such a formulation was not adequate for treatment of PKU.

The current study with LNAA gave consideration to the transport of Phe in the GI tract, where the K_m of the carrier protein in the GI tract is two orders of magnitude higher than that in the BBB. It is interesting that lysine and arginine are also transported by the same carrier protein (Hidalgo and Borchardt 1990; Larsen et al 1964; Pardridge 1982). Experiments by Hidalgo and colleagues (1990) using human intestinal-epithelial cells, Caco-2-cells, in monolayers with a buffer containing 10 µmol/L Phe, showed significant inhibition of Phe transport requiring 100-fold (1 mmol/L) LNAA, as dictated by the K_m equation for affinity of LNAA to the GI carrier protein. For example, at such concentrations, leucine inhibits Phe transport by 55%, tyrosine by 45% and the cationic amino acid lysine by 50%. Competition of LNAA with Phe is likely to occur in the GI tract only if LNAA is given in high concentration. We have shown in PKU mice that when 16.7% of LNAA was added to the normal chow a statistically significant decrease in blood Phe concentration was observed (Matalon et al 2003.)

The success of the open-label trial with LNAA (Matalon et al 2006) in lowering blood Phe concentration in patients with PKU suggests that the carrier protein of the GI tract can be inhibited in the transport of Phe to the blood. The results of the double-blind study clearly show a significantly lowering of the blood Phe concentration while on LNAA (NeoPhe). The reduced concentration of Phe was found in subjects from six participating metabolic centres. The lower blood Phe concentration was observed in seven patients with classical PKU who were on protein-free food and PKU formula, who had an average blood Phe concentration of 531.6 µmol/L which dropped to 281.5 µmol/L (average drop of 250.1 µmol/L, SD $= \pm$ 173.7, $p = 0.009$) (Fig. 1). The reduction of blood Phe in the seven patients was 47%, which is statistically significant.

It is possible that LNAA may contribute to better utilization of Phe, or another anabolic effect. However, an anabolic effect has not been observed in long-term treatment of LNAA in mice with PKU.

It is important to underscore, that LNAA can lower blood Phe concentration in all patients with PKU. Such results are not likely to occur on treatment with BH_4, where the response is mainly in those with mild or atypical PKU. Therefore, LNAA offer a new modality of treatment of PKU when routine treatment with protein-free food and PKU formula are not successful in lowering blood Phe concentrations.

The data presented suggest that long-term studies with LNAA (NeoPhe) are required to establish safety, long-term efficacy and long-term compliance. The number of pills seems high; it is now possible that, with improved technology from the food industry, LNAA can be given in powder form, or chewable forms, and be made more palatable, so that taking LNAA will be more acceptable to patients.

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EXHIBIT D

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ABSTRACTS

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Exhibit D

041-P

EFFECT OF LNAA ON BLOOD PHENYLALANINE IN PKU

R Matalon[1], K Michals-Matalon[2], J Nsen[3], K Ahring[3], L Brammer[3], P Novikov[4] E Grechanina[5]

University of Texas Medical Branch, Galveston, TX[1], University of Houston, Houston, Texas[2], USA; Kennedy Institute, Glostrup, Denmark[3], University of Moscow, Russia[4], Center for Genetics, Kharkiv, Ukraine[5]

Studies with large neutral amino acids (LNAA) in mice with PKU (ENU2) showed decrease in blood phenylalanine (Phe) when LNAA (PreKUnil) was given orally (Matalon, R. et al. Pediatr. 112:1570, 2003). While previous studies using LNAA were focused on blood brain barrier, this study was aimed to evaluate the effect of LNAA on the transport of Phe in the GI tract. PreKUnil is deficient in lysine, and its use in the treatment of PKU has resulted in negative nitrogen balance. A new formula of LNAA with adequate lysine, NeoPhe, taking into account the Km of these amino acids and their affinity for GI transporter was used in the present study. Subjects were chosen by the clinical directors of 4 different sites. There were 14 patients with PKU whose mean baseline blood Phe was 1266 µmol/L who were given 0.5 g/kg NeoPhe per day in 3 divided doses taken with meals. Blood Phe was drawn twice weekly and then weekly, thereafter. After two days the mean blood Phe decreased to 1073 µmol/L and at one week 869 µmol/L. The mean decline in blood Phe was 32% at 1 week. One subject had no change in blood Phe. The NeoPhe was tolerated and no untoward effects were reported during the trial. This pilot study indicates that LNAA can lower blood Phe in patients with PKU, especially those who have high concentrations of blood Phe. Because of the reduction of blood Phe, LNAA can compete more effectively with Phe for transport to the brain, which may have implications on brain development, and neurotransmitter concentrations.

042-P

INCREASED TOLERANCE OF PHENYLALANINE IN THE TREATMENT OF PHENYLKETONURIA (PKU) WITH THE USE OF LARGE NEUTRAL AMINO ACIDS

K Moseley[1], MD Brown[2], S Yano[1], R Koch[1]

[1]Childrens Hospital Los Angeles, Division of Medical Genetics, Los Angeles, California; [2]Dixie Regional Medical Center, St. Ge, Utah, USA

We report a relevant finding with one reliable subject who is 30 years old, is in the medical field and has been followed in our clinic since birth. This individual has classic PKU with the mutations R243X/IVS1nt5g>t and a normal IQ. The phe-restricted diet was initiated shortly after birth with blood phe concentrations kept under 10 mg/dl. Phe intake for the past 10 years has been approximately 300 mg/d. Recently, this subject stated that with age it was becoming increasingly difficult to maintain blood phe concentrations under 10 mg/dl despite the use of low protein products. It was decided that PreKUnil would be used as an adjunct therapy along with the medical food product but in a lower dose. The usual meal plan includes approximately 50 g protein from a medical food product (6 Phlexy-10 sachets), 30–40 g protein from natural sources and 3–4 PreKUnil tablets at each meal for a total of approximately 1.6 g protein/kg body weight. The phe from natural foods has increased to approximately 1400–2000 mg/d and blood phe concentrations have remained under 12 mg/dl for the past year. This subject also reports feeling more energetic and 'clear headed' since using the tablets. We have noticed a similar trend in our other patients on the PreKUnil tablets. There is a definite increase in blood tyrosine and tryptophan in individuals using the PreKUnil tablets, which may indicate a better metabolic balance of the amino acids in the sources of protein.

EXHIBIT E

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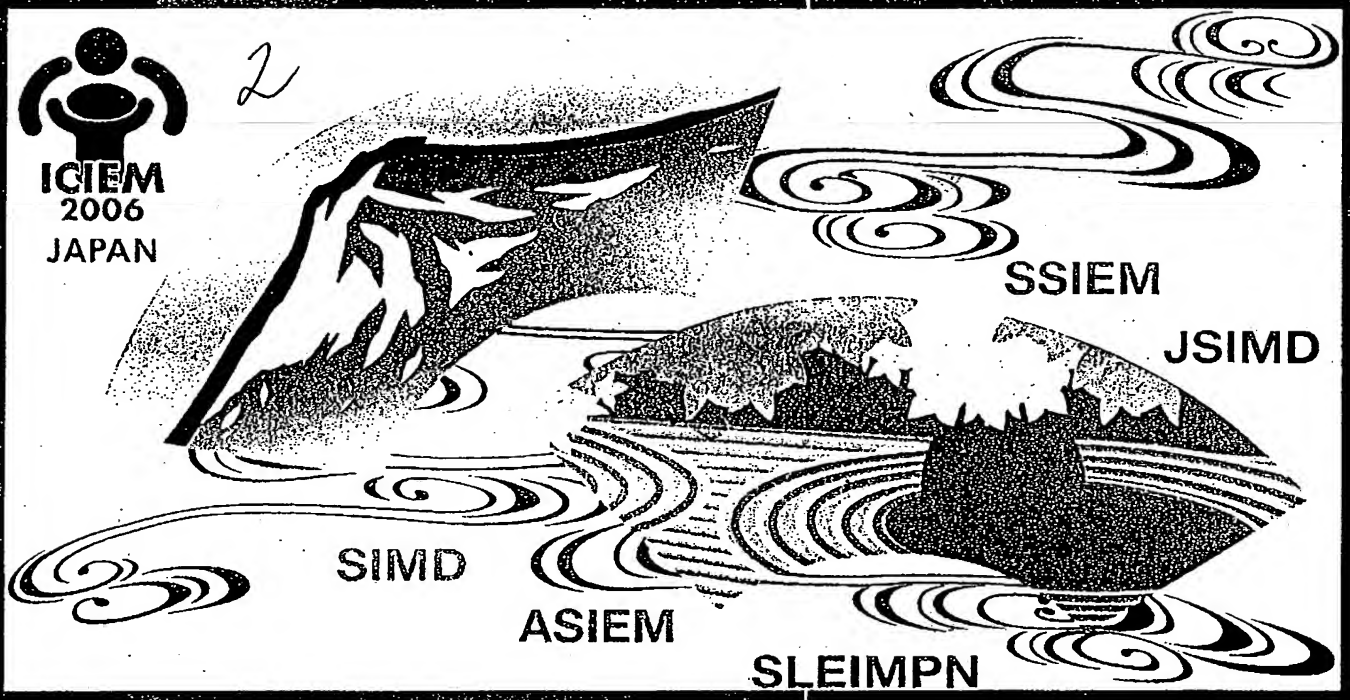

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Exhibit E D14

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WS-1-1

FREQUENCY OF *PAH* MUTATIONS IN BH_4-RESPONSIVE PKU

Zurflüh M[1], Thöny B[1], Zschocke J[2], Blau N[1]
[1]*Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zürich, Switzerland;* [2]*Institute of Human Genetics, University of Heidelberg, Heidelberg, Germany*

Patients with BH_4-responsive PKU respond to BH_4 by lowering their blood Phe levels 8–24 h after oral administration (20 mg/kg). Based on the BH_4 loading test, an frequency of BH_4-responsiveness was calculated to be 60–70% in mild HPA/PKU, ~30% in moderate PKU, and ~5% in classic PKU (overall ~40%). Most of BH_4-responsive patients are compound heterozygotes, carrying at least one mutant allele with residual phenylalaine hydroxylase (PAH) activity, 29 are homozygotes for two active alleles, but none was homozygote or compound heterozygote for two null mutations. In order to estimate the population of BH_4-responsive PKU patients on basis of the genotype, we established a database of *PAH* mutations BIODEF (www.bh4.org).

So far 264 patients with BH_4-responsive HPA/PKU and 519 alleles with 112 different mutations are tabulated in the database. 340 alleles (66%) are located in the catalytic domain, 74 (14%) in the regulatory domain, 53 (10%) in the tetramerization domain, and 52 (10%) are intornic. 316 alleles (61%) exert residual PAH activity (~43% compared with the wt enzyme), 29 alleles (5%) are potentially active, 87 alleles (17%) are inactive, and 87 alleles (17%) are not yet defined. The most common BH_4-responsive mutations (occurring in >10 alleles) are A403V (8.5%), R261Q (6.9%), Y414C (6.7%), V245A (4.7%), A300S (4.7%), R241C (4.0%), I65T (3.6%), E390G (3.6%), V388M (2.4%), and L48S (2.2%).

WS-1-2

A PHASE 3 STUDY OF THE EFFICACY OF SAPROPTERIN IN REDUCING PHE LEVELS IN SUBJECTS WITH PHENYLKETONURIA

Levy H[1], Milanowski A[2], Chakrapani A[3], Cleary M[4], Trefz F[5], Whitley C[6], Feillet F[7], Feigenbaum A[8], Bebchuk J[9], Christ-Schmidt H[9], Dorenbaum A[10]
[1]*Children's Hospital of Boston, Boston, MA, USA,* [2]*Instytut Matki i*

WS-1-3

DOUBLE BLIND PLACEBO CONTROL TRIAL IN PKU WITH NeoPhe

Matalon R[1], Michals-Matalon K[2], Burlina A[3], Burlina A[3], Giovannini M[4], Fiori L[4], Grechanina E[5], Novikov P[6], Grady J[1], Tyring S[7], Guttler F[8]
[1]*University of Texas, Galveston, TX, USA.* [2]*University of Houston, Houston, TX, USA,* [3]*University of Padova, Padova, Italy;* [4]*University of Milan, Milan, Italy,* [5]*University of Kharkiv, Kharkiv, Ukraine,* [6]*University of Moscow, Moscow, Russia,* [7]*University of Texas, Houston, TX, USA,* [8]*Kennedy Institute, Glostrup, Denmark*

Studies in our center with large neutral amino acids (LNAA), resulted in decrease in blood phenylalanine(phe). The initial formula, PreKUnil, was deficient in lysine. New formula of LNAA, NeoPhe, with lysine was made. The decrease in blood phe concentration using NeoPhe was documented in mice and patients with PKU. One week, double blind placebo controlled trial was conducted with NeoPhe. Sixteen patients were enrolled from six different centers in the US, Italy, Ukraine and Russia. The average baseline of phenyalanine in these patients was 1120 µmol/L. Patients were given 0.5 g/kg of NeoPhe or placebo tablets, in 3 divided doses, taken with meals. Baseline blood phe was determined prior to the trial, then every other day when on treatment. There was significant blood phe drop in patients on NeoPhe, which averaged 27% from baseline levels. During the placebo trial, blood phe did not vary significantly, and in some cases, blood phe went slightly higher. This double blind study indicates that LNAA can compete with phe on the transporter in the GI tract. The number of NeoPhe tablets to be taken should be adjusted to the needs and the blood phe concentrations of each patient. Longer term study of NeoPhe and placebo needs to be conducted in order to establish the efficacy and tolerance of NeoPhe in long term treatment of PKU. These results are encouraging as more LNAA can cross the blood brain barrier since blood phenylalanine is lowered, which should result in improved neurotransmitter concentrations of PKU patients.

WS-1-4

LONG-TERM TREATMENT OF TETRAHYDROBIOPTERIN (BH_4)-RESPONSIVE MILD PKU IN JAPAN

Shintaku H[1], Ohura T[2], Shyoji Y[3], Kobayashi H[4], Ohwada M[5], Yamano T[1], Aoki K[6], Kitagawa T[7]
[1]*Dept. of Pediatrics, Osaka City University Graduate School of Medicine, Osaka, Japan,* [3]*Dept. of Pediatrics, Tohoku University*

EXHIBIT F

JIMD

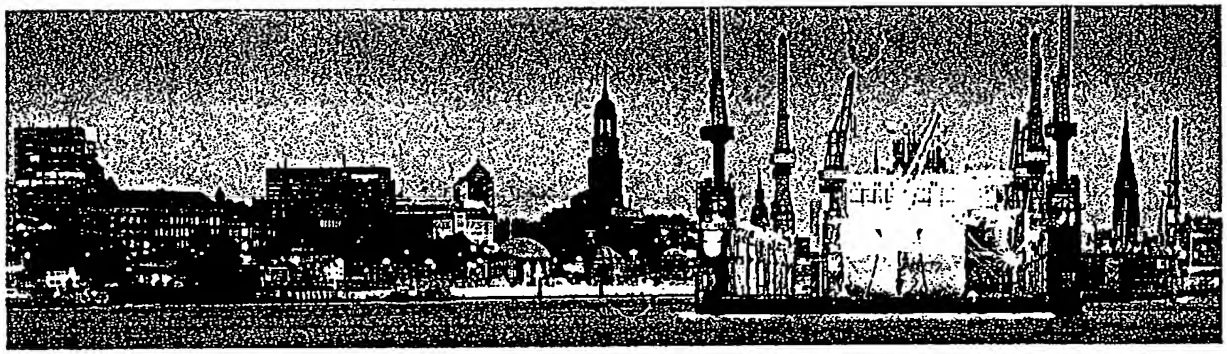
Journal of Inherited Metabolic Disease

Volume 30 · Suppl. 1 · August 2

SSIEM 2007 3

Annual Symposium

Society for the Study of Inborn Errors of Metabolism



Annual Symposium of the Society for the Study of Inborn Errors of Metabolism

Hamburg, Germany

4–7 September 2007

Abstracts

Springer SSIEM

www.jimd.org

Exhibit F D15

049-P

WHAT HAPPENS WITH A LARGE BOLUS OF AMINO ACIDS IN PKU PATIENTS?

Hoeksma M[1], Reijngoud DJ[2], van Rijn M[1], Szczerbak B[3], Gross M[3], van Spronsen FJ[1]
[1]Beatrix Child Hosp, UMCG, Univ Groningen, Groningen, Netherlands, [2]Lab Metab Dis, UMCG, Univ Groningen, Groningen, Netherlands. [3]Milupa, Friedrichsdorf, Germany

Background/Objective: Compliance to dietary prescriptions is important in treatment of amino acid IEMs. Division of the amino acid mixture (AAM) over three or more portions per day is recommended, but in daily practise patients take the AAM in one portion. In order to give evidence-based advise for the distribution of the AAM over the day, the utilization was studied in well controlled PKU patients and controls, receiving the AAM (PKU 3, Milupa, Germany) in one bolus. Methods: 4 adult PKU patients and 2 healthy adults were studied. They consumed a bolus (0.8*1.2 g/kg), representing 100% of daily prescribed AAM. Stable isotope kinetics of ^{13}C-valine and $NaH^{13}CO_3$ were used to study whole-body protein metabolism before and after intake of AAM.

Results:

Time (min)	Infuse rate (μmol/kg/h)	Rate of appearance of Valine (μmol/kg/h) Controls	PKU	Valine oxidation rate (μmol/kg/h) Controls	PKU
Fasting	t<0	7.36 (0.12)	97 (10)	110 (13)	23 (4)	23 (3)
Post prandial-	15	10.71 (0.82)	136 (24)	165 (23)	22 (10)	25 (5)
	60	18.79 (0.90)	240 (6)	267 (22)	35 (3)	33 (4)
	120	15.24 (0.53)	193 (26)	217 (35)	55 (9)	55 (9)
	180	11.17 (0.51)	158 (18)	154 (22)	68 (11)	69 (10)
	270	7.70 (0.21)	104 (10)	110 (17)	71 (8)	67 (12)

Thirty-eight and 32% of the bolus was oxidised at t = 270 min in controls and PKU's, respectively. Maximum estimated protein synthesis rate (uncorrected for time delays) was reached 60 min after bolus ingestion. Conclusions: Synthesis rate seems influenced by plasma concentration. Oxidation starts rather slowly but contributes largely to disappearance. Amino acid metabolism is similar in PKU patients and healthy controls. The effect of distributing the AAM-bolus will be investigated.

050-P

EXPERIENCE WITH LONG TERM USE OF LNAA IN TREATMENT OF PKU

Matalon R[1], Michals-Matalon K[2], Bhatia G[1], Grady J[1], Tyring S[1]
Univ of Texas Medical Branch, Galveston, United States, [2]Univ Houston, Houston, United States

Previous loading of short term studies with Large Neutral Amino Acids (LNAA) in PKU patients resulted in decrease of blood phenylalanine (Phe) levels. The long term safety, efficacy and acceptability of LNAA tablets (NeoPhe) have not been evaluated. In this study, four patients, three female and one male, ages 25 to 38 years, were given NeoPhe tablets, 0.5 g/kg/day in three divided doses to be taken with meals. The patients were not on medical foods, for more than 10 years previously. Their blood Phe prior to taking NeoPhe had a mean value of 1507 μmol/L. Blood Phe was determined two weeks after entering the study and once a month for a period of 12 months. The mean blood phe level declined for each of the subjects during the study period: 642 μmol/L, 707 μmol/L, 899 μmol/L and 869 μmol/L. The mean change from pre- to post-NeoPhe was significant (paired t-test: p = 0.002). Patients reached levels within NIH recommendations. Patients were monitored for weight in case, LNAA was used for protein synthesis. None of the patients gained or lost any weight beyond minor fluctuation of ±0.2kg. The acceptability of the pills was monitored with review on every visit and there were no complains regarding the number of pills or of abdominal discomfort, nausea or changes in bowel habits. All patients asked to continue taking NeoPhe tablets because they were happy with their blood phe levels and indicated they felt 'more focused' at work. Future studies should include larger number of patients and neuropsychological tests need to be added.

051-P

THE EXPERIENCE WITH LARGE NEUTRAL AMINO ACIDS IN UKRAINE

Grechanina OYa[1], Matalon RK[2], Novikova IV[3], Fedoseeva NP[3], Zdibskaya OP[1], Grechanina YuB[1]
[1]Kharkiv State Med Univ, Kharkiv, Ukraine, [2]Univ Texas Med Branch, Texas, United States, [3]Kharkiv Spec Med Gen Cent, Kharkiv, Ukraine

Kharkiv Specialized Medical Genetic Centre (KhSMGC) took part in collaborative study with Texas University (Prof. R. Matalon) on using of new formulas (Large Neutral Amino Acids (LNAA)) for PKU patients in Ukraine. LNAA have been used with the purpose to decrease the influx on phenylalanine (Phe) to the brain.

The mixture of LNAA (NeoPhe) was obtained from Prekulab, Korsör, Denmark. Patients with PKU were observed at KSMGC. NeoPhe was given 1 pill/kg/day in 3 doses. For biochemical control of Phe level we used fluorometric assay and TLC; for confirming diagnostics – HPLC; PCR – for mutation analysis. Patients were instructed to continue their diet as they did before the trial. Baseline was determined before and after taking NeoPhe.

Clinical approbation of NeoPhe in five PKU patients at the age of 11–22 years (mean 15.2) was performed. There were revealed such genotypes: R408W/R252W, R408W/R408W, R408W/R408W, R261X/R408W. The mean Phe level before NeoPhe administration was 1143.2 μmol/L; after taking NeoPhe – 739.3 μmol/L. We have detected the decreased mean Phe blood level by 35.3%.

Our data indicate that the inhibition of transport of Phe may occur in the GI tract using LNAA. The decrease of blood Phe levels in PKU patients taking LNAA was reported in Ukraine at the first time. It is possible if natural protein is limited (less Phe), more effective competition of LNAA with Phe might occur, that leads to lower blood Phe concentrations.

052-P

THE BLOOD/BRAIN BARRIER IN NEONATES: ^{1}H-MR SPECTROSCOPY SHOWS LOW PROTECTION AGAINST HIGH PHENYLALANINE

Nuoffer JM[1], Trapp-Chiappini D[2], Zwygard K[3], Bösch Ch[3], Pietz J[4], Kreis R[3]
[1]Div Metab Dis, Univ Child Hosp, Berne, Switzerland, [2]Inst Clin Chem, Univ Hosp, Berne, Switzerland, [3]Dept Clin Res, Univ Hosp, Berne, Switzerland, [4]Pediatr Neurol, Univ Child Hosp, Heidelberg, Germany

Background: ^{1}H-MRS has been used to determine the blood/brain ratio for Phe in adults PKU patients. While the exact value is still debated, there is consensus that brain Phe is 3–4 times lower than in blood. The most crucial time for outcome is early childhood but data on blood/brain ratio for Phe in newborns are not available.

Objective: To determine the blood/brain ratio for Phe in neonates with PKU.

Methods: All spectra were recorded on a 1.5 TMR scanner. So far 2 neonates with PKU were investigated: Patient A, 43 weeks gestational age (GA), at 9 days; Patient B 36 weeks GA at 9 and 14 days. Controls: 2 healthy neonates 43 and 44 weeks GA. Adults: 6 PKU patients (23 ± 8 y old) and 6 healthy subjects.

Results: ^{1}H-MRS spectra were all of good quality. The blood/brain ratio is strikingly higher for neonates (0.52–1.2) than adults (0.29 ± 0.04). After treatment Phe droped to normal in parallel with blood levels in patient B, such that their ratio (1.2) is ill-defined.

Discussion: This study shows that the blood–brain barrier does not provide the same protection against high Phe for newborns as it does for the adults. At identical blood Phe levels newborn PKU patients' brain is exposed to much higher Phe level than adults. This underlines the importance of strictest dietary control in young age.

EXHIBIT G

NeoPhe in the Treatment of Phenylketonuria

New Formulation of LNAA

SSIEM

Paris, France

September 2005

Exhibit G

USA

Reuben Matalon, M.D., Ph.D.

Kim Matalon

Russia

Peter Novikov

Denmark

Ukraine

Elena Grechanina

Transport of LNAA to the Brain

	K_m mmol/L	K_m app
• Phenylalanine (Phe)	0.12	0.45
• Leucine	0.15	0.53
• Tyrosine	0.16	0.58
• Tryptophan	0.19	0.71
• Methionine	0.19	0.77
• Histidine	0.28	1.10
• Isoleucine	0.33	1.30
• Valine	0.63	2.50
• Threonine	0.73	3.00

Pardridge, Inborn Errors of Metabolism in Humans.
MTP Press, 1980.

Andersen AE, Avinsl

- LNAA injected to rat pups
- Phenylalanine hydroxylase was ihibited by parachlorophenylalanine
- Brain phenylalanine decreased

1976 Arch Neurology 33:684

Tyrosine in The Treatment of PKU

Lou et al used Tyr 160 mg/kg in treated patients with PKU

- Increased attention span
- Increased dopamine synthesis

1987 Acta Paediatr Scand 76:560

Tyrosine in Treatment of PKU

- Pietz et al. used high dose tyrosine in adults with PKU and high blood Phe
- No difference in treated group vs placebo

1995 J Pediatr 127:936

Tryptophan in Treated PKU

- Nielsen et al used tryptophan 4.5 gm/day to treated PKU for 3 weeks
- Showed a 3 fold increase in 5-HIAA in CSF despite high blood Phe

1988 Dietary Phenylalanine and Brain Function. Birkhauser

$$Km\ (app) - Km\ (1 + \sum[aa]/Km]$$

This predicts that, if the plasma level of an LNAA is much less than its value of Km, then that amino acid will not compete effectively for the carrier protein

Absolute and apparent Km values of neutral amino acids for the neutral amino acid transporter in the BBB (Partridge, 1980)

Amino acid	Typical plasma level (mM)	Km (mM)	App Km (mM)
LNAA's			
Phe	0.05	0.12	0.45
Leu	0.10	0.15	0.53
Tyr	0.09	0.16	0.58
Trp	0.10	0.16	0.71
Met	0.04	0.19	0.77
Isoleu	0.07	0.33	1.3
Val	0.14	0.63	2.5
Thr	0.19	0.73	3.0
Basic aa's			
His	0.05	0.28	1.1
Arg	0.10	0.09	0.40
Lys	0.30	0.10	0.25

LNAA Transport in Intestinal Mucosa K_m mmol/L

- Phenylalanine 1.0
- Leucine 2.0
- Valine 3.0
- Methionine 5.0
- Histidine 6.0
- **Competition effect is not likely to occur in tissue other than brain unless high concentration of amino acids is used**

Pardridge, Inborn Errors of Metabolism in Humans. MTP Press, 1980.

Amino acid inhibition of Phe transport in Caco-2-cells – 10uM Phe in buffer applied to monolayers in presence of 1 mM concentration of each amino acid

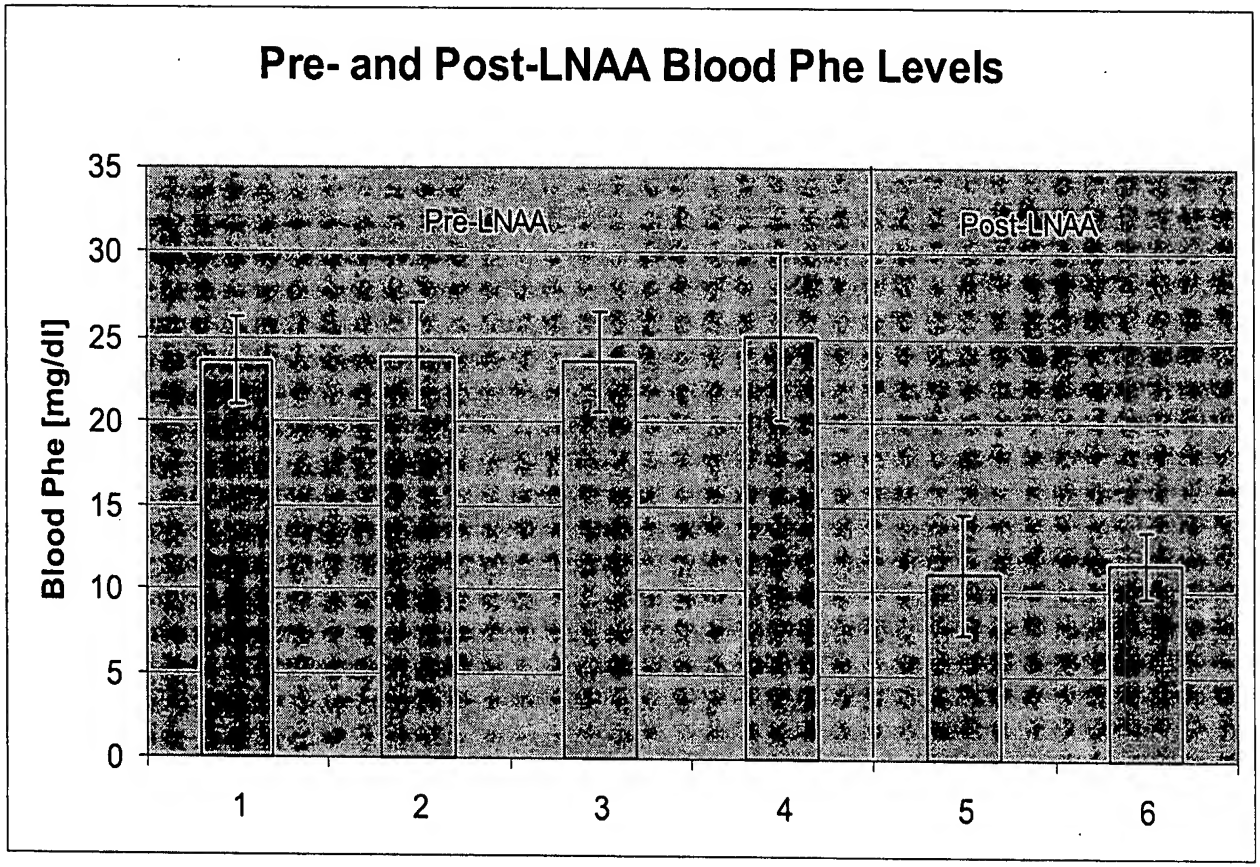
Inhibitor	% inhibition
LNAA's	
Leu	55%
Tyr	45%
Trp	36%
Basis Aa's	
Lys	50%
His	33%

Hidalgo Biochem Biophys. Acta 1008: 5-30a (1990)

PKU Mice on NeoPhe Control

				phe mg/dl	NeoPhe	
#	78-04	80-04	83-04	86-04	159-04	162-04
F 577	23.8	21.4	19.4	24.9	18	11
F 579	23.7	25.1	28.4	33.1	8.3	14.8
F 582	28.8	21.9	22.2	20.9	10.3	8.3
F 584	23.8	30	25.3	30.7	7.8	12.4
F 585	20.6	21.7	24.4	19.8	8.5	12.3
F 586*	23.2	25.7	21.2	21.9	13.5	11.3
F 588	21.6	21.7	24.2	24.4	10.9	10.9
Avg each time pt	23.6	23.9	23.6	25.1	11	11.6
Avg all Pre-LNAA	24.1					
Avg all Post-LNAA	11.3					

*Pre-exposure to 16.7% LNAA


Pre- and Post-LNAA Blood Phe Levels
Blood Phe [mg/dl]
35
30
25
20
15
10
5
0
Pre-LNAA
Post-LNAA
1
2
3
4
5
6

Denmark LNAA STUDY

Blood Samples	PKU 20	PKU 39	PKU 93	PKU 105	PKU 128	Average
1 tablet/kg 01	1436	1681	1697	1597	1627	1608
02	1262	1691	1591	1480	1602	1525
04	1164	1643	1526	1414	1407	1431

Denmark LNAA STUDY

2 tablets/kg 08	1252	1739	1477	1413	1359	1448
09	1146	1537	1370	1233	1373	1332
11	1119	1556	1389	1179	1313	1311
15	1199	1650	1349	1222	1335	1351
Decrease after 1 week	184	-58	220	184	268	160
Decrease after 2 week	237	31	348	375	292	257

Russia LNAA STUDY

	Phe		Tyr	
KA Time	µmol/l	mg/dl	µmol/l	mg/dl
0’	718.8	11.98	53.9	0.98
3 days	668.4	11.14	91.3	1.66
3 days	523.2	8.72	103.4	1.88
3 days	376.2	6.27	108.3	1.97
KN Time	µmol/l	mg/dl	µmol/l	mg/dl
0’	707.4	11.79	42.9	0.78
3 days	607.2	10.12	126.5	2.30
3 days	572.4	9.54	159.5	2.91
3 days	585.6	9.76	83.6	1.52

Russia LNAA Study

	Phe		Tyr	
KH Time	µmol/l	mg/dl	µmol/l	mg/dl
0’	635.4	10.59	33.0	0.60
3 days	554.4	9.24	242.0	4.40
3 days	322.2	5.37	94.6	1.72
3 days	136.2	2.27	110.0	2.00
3 days	102.6	1.71	94.0	1.71

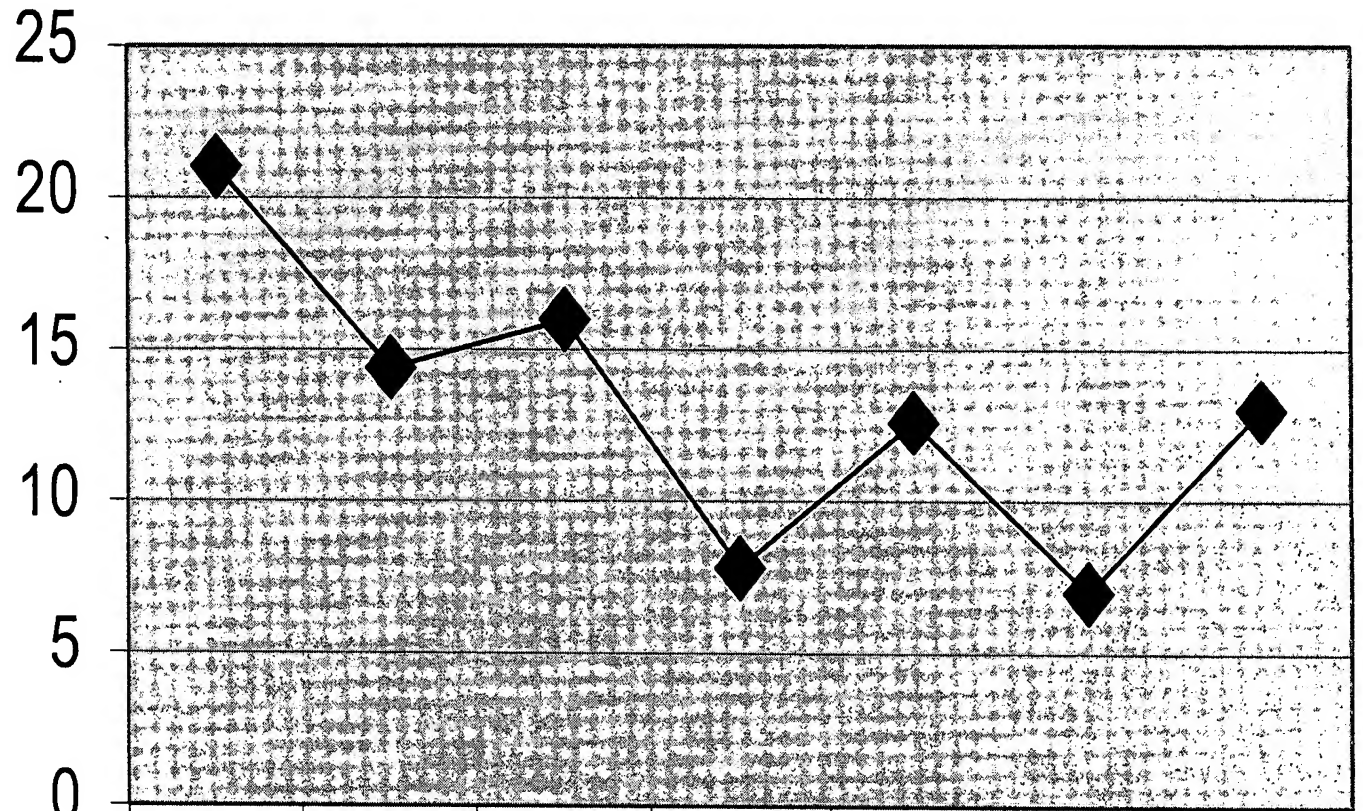
USA LNAA STUDY

	Phe		Tyr	
GDL Time	µmol/l	mg/dl	µmol/l	mg/dl
0'	1290.6	21.51	69.8	1.27
2 days	1198.2	19.97	73.7	1.34
4 days	115.8	1.93	140.25	2.55
KM Time	µmol/l	mg/dl	µmol/l	mg/dl
0'	1540.2	25.67	30.8	0.56
8 days	883.8	14.37	53.8	0.98
0'	1978.2	32.97	68.7	1.25
2 days	1608.6	26.81	207.35	3.77

USA LNAA STUDY

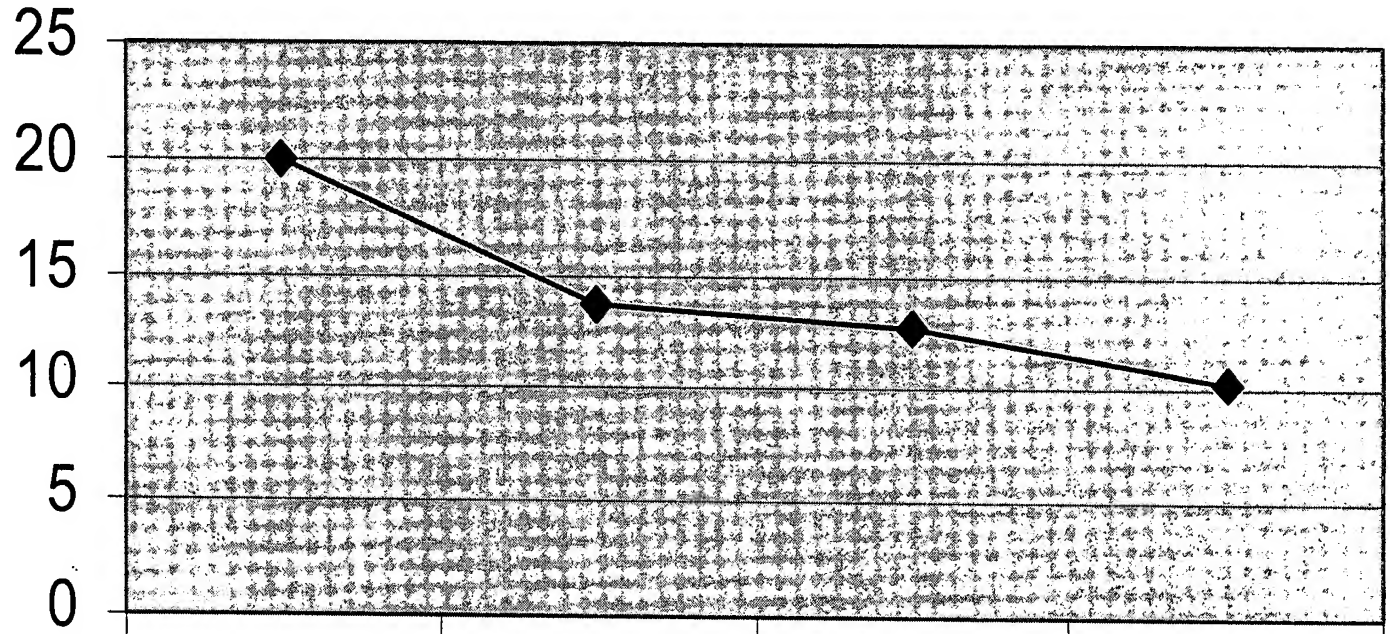
	Phe		Try	
ES Time	µmol/l	mg/dl	µmol/l	mg/dl
0’	1375.8	22.93	31.9	0.58
4-7 days	767.4	12.79	121.5	2.12
RC Time	µmol/l	mg/dl	µmol/l	mg/dl
0’	965.4	16.09	58.8	1.07
2 days	828.6	13.81	156.2	2.84

Response of Blood Phe to LNAA
Ukraine



	0	24h	72hr	1 wk	2 wk	3 wk	4 wk
21 yo girl	21	14.5	16	7.9	12.7	7.1	13

Response on Phe on LNAA
Ukraine



	0	36hr	72h	1wk
12 yo boy	20	13.8	12.75	10.4

1200 **828** **765** **624**

US Blood Phe and Tyr
NeoPhe Patient K 1 Week
µmol/L (mg)

Control			NeoPhe		
phe		tyr	phe		tyr
			µmol/L (mg)		
1978.1	32.97	1.25	1356.0	22.6	5.0
1139.6	25.66	0.62	1308	21.8	4.1
1456.2	24.27	0.62	1146	19.1	3.82

24% reduction

US Blood Phe and Tyr
NeoPhe Patient G 1 Week

Control			NeoPhe		
phe		tyr	phe		tyr
	mg/dl			mg/dl	
1560	26.0	0.92	953	15.89	4.35
1764	29.4	1.9	505	8.43	3.32

56% reduction

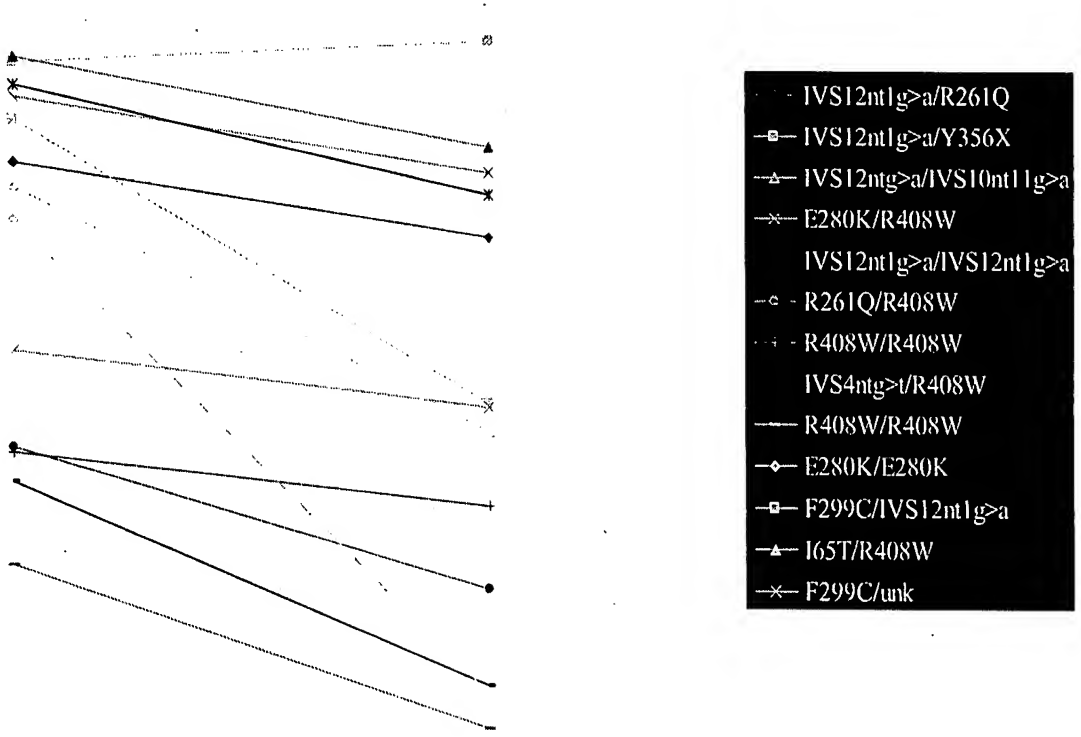
NeoPhe 0.5 g/kg in PKU Subjects

- 13 subjects
- Mean age 26.6 years
- 7 males, 6 females
- Mean decrease in blood Phe after one week 243 μmol/L
- Average decrease in blood Phe 22 %.

NeoPhe 1.0 g/kg in PKU Subjects

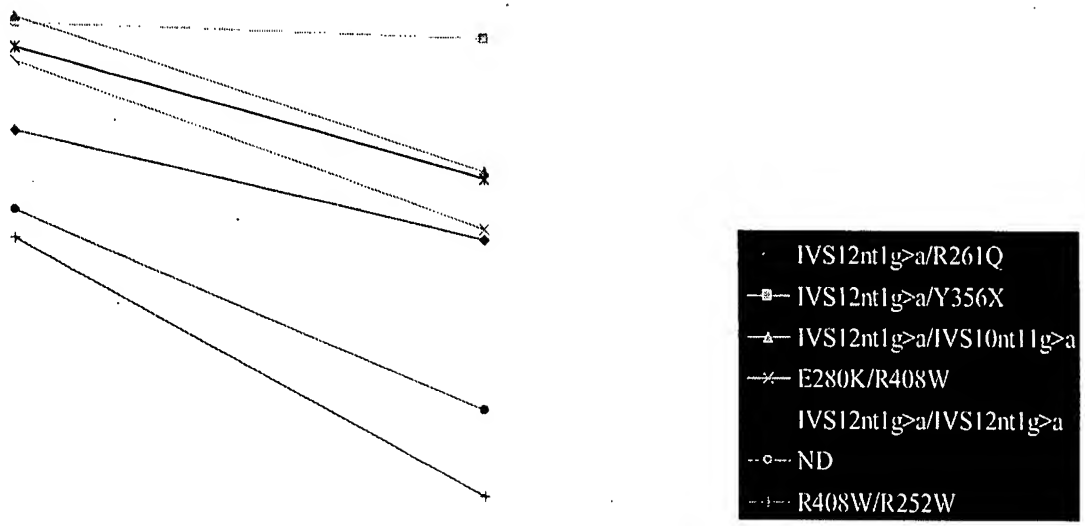
- 7 subjects
- Mean age 25.2 years
- 5 males, 2 females
- Mean decrease in blood Phe after one week 377 μmol/L
- Average decrease in blood Phe 25 %.

Figure 1. Blood Phe Response to 0.5g/kg NeoPhe in Patients with PKU



Paired t-test: p=0.001

Figure 2. Blood Phe Response to 1.0 g/kg NeoPhe in Patients with PKU



Paired t-test: p=0.006

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the Genetics Research trust.

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acknowledge to **Bent Holm** from
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